SEROTONERGIC AND HYPOCRETINERGIC SYSTEMS MODULATE VENTILATION AND HYPERCAPNIC VENTILATORY RESPONSES

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SEROTONERGIC AND HYPOCRETINERGIC SYSTEMS MODULATE VENTILATION AND HYPERCAPNIC VENTILATORY RESPONSES

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Abstract

Serotonergic (5-HT) cells of the medullary raphé are putative central chemoreceptors, one of multiple chemoreceptive sites in the brainstem that interact to produce the respiratory chemoreflex. This role is debated, and the importance of 5-HT neurons as chemoreceptors in relatively intact systems is unclear. The main focus of this dissertation is to provide further physiological evidence for the involvement and modulation of 5-HT neurons in CO_2 chemosensitivity. This is of interest as a large number of Sudden Infant Death Syndrome (SIDS) cases report dysfunction in the 5-HT system, and CO_2 may be an exogenous stressor leading to SIDS when in combination with this underlying vulnerability. Also, since SIDS occurs primarily during sleep, I also focus on the potential functional interaction between the 5-HT and hypocretinergic systems, as hypocretins play a role in arousal and also potentially in chemosensitivity. I confirm the hypothesis that the serotonergic and hypocretinergic systems modulate ventilation and hypercapnic ventilatory responses. Using the *in situ* preparation derived from juvenile rats and the *in vitro* medullary slice preparation from mice, I verify that 5-HT neurons are critical in generating a response to CO_2, primarily via facilitation of the respiratory rhythm through $5\text{-}HT_2$ receptors. I also find evidence to support the hypothesis that hypocretins play a significant role in the neuroventilatory response to CO_2 through activation of hypocretin receptors type 1. By comparing results from rhythmic medullary slice preparations from wildtype (normal 5-HT function) and $Lmx1b^{f/f/p}$ (lack central 5-HT neurons) neonatal mice, I attempt to identify whether changes in hypoglossal nerve output in response to acidosis are affected by hypocretin receptors, and whether this is dependent on the presence of 5-HT neurons. Frequency results from such studies are inconclusive; however, hypocretins do appear to mediate the burst duration response via serotonergic mechanisms. I also find that hypocretins facilitate baseline neural ventilatory output in part through 5-HT neurons. Thus, both the 5-HT and hypocretinergic systems are involved in modulating ventilation and hypercapnic ventilatory responses.

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Chapter 1 General introduction

1.1. Control of breathing

Breathing in mammals is generated in the brainstem. Neurons with respiratory-related activity are organized in a ventrolateral column known as the ventral respiratory group (VRG). Two sites have been identified as putative central rhythm generators for breathing, containing neurons with pacemaker-like properties that contribute to maintaining such a rhythm. The preBötzinger Complex (preBötC) is located at the rostral end of the VRG, is both necessary and sufficient for breathing, and appears to drive inspiratory activity (Smith et al. 1991). More recently, the parafacial respiratory group (pFRG) was identified as a group of neurons just rostral to the facial nucleus that fire ~500 msec before inspiratory activity of the preBötC occurs. Neurons of the pFRG appear to drive active expiratory active (Feldman and Del Negro 2006). The preBötC and pFRG appear to act together as a coupled oscillator to produce the respiratory rhythm (Feldman and Del Negro 2006; Onimaru and Homma 2003). Other nuclei in the VRG, including the retrotrapezoid nucleus, Bötzinger complex, nucleus of the solitary tract, adrenergic nuclei, and raphé nuclei, modulate the pattern of the respiratory rhythm through release of various neuromodulators (Fig. 1.1). Neurons of the VRG are anatomically linked to motor neuron pools in order to activate respiratory muscles that cause ventilation (Feldman et al. 2003).

Circuits generating breathing are modified by specific inputs in order to match ventilation to metabolic demands. Examples of such modulation are via feedback from chemoreceptors. Populations of chemoreceptors in the periphery, carotid and aortic bodies, respond to fluctuations in blood gas compositions and initiate ventilatory responses to return blood gases to homeostatic levels (Day and Wilson 2009). Other populations are located within the central nervous system (central chemoreceptors) (Day and Wilson 2009; Feldman et al. 2003). The nature of central chemoreception is debated and a topic of this dissertation. As will be described, serotonin-synthesizing neurons of the raphé have been proposed as important central chemoreceptors.

Breathing and chemosensitivity exhibit arousal state-dependent changes between sleep and wakefulness (Day and Wilson 2009; Dias et al. 2009; Gestreau et al. 2008). The hypocretinergic system contributes to arousal state-mediated influences on homeostasis (Dias et al. 2009; Gestreau et al. 2008). Hypocretin and serotonin systems are anatomically connected (Day and Wilson 2009; Dias et al. 2009; Gestreau et al. 2008). Therefore, I propose that state-dependent changes in breathing and chemosensitivity may be explained by hypocretin influences on 5-HT-mediated processes.

1.2. The raphé nuclei

The raphé nuclei can be divided into two major groups, rostral and caudal. The rostral group is located in the midbrain, includes the dorsal raphé, and projects rostrally to the forebrain playing roles in arousal, cognition, and mood (Dahlstrom and Fuxe 1964; Hornung 2003; Jacobs and Azmitia 1992). The caudal group is located predominantly in the medulla and comprises midline nuclei such as the raphé pallidus, raphé magnus, and raphé obscurus in addition to the parapyramidal region located at the ventrolateral medullary surface. These nuclei project to other brainstem nuclei and the spinal cord (Dahlstrom and Fuxe 1964; Hornung 2003) and are involved in pain modulation, temperature regulation, sexual function, cardiovascular control, and breathing (Mason 2001; Morrison 2004). The raphé nuclei form part of the reticular system and are composed of mixed populations of neurons. However, the vast majority of neurons synthesizing serotonin (5-hydroxytryptamine, 5-HT) in the central nervous system are located within the raphé (Hilaire and Duron 1999). Although not primarily a respiratory structure, the raphé has been linked to ventilation. Many raphé neurons project to ventilatory motor control areas such as the hypoglossal and phrenic nuclei (Holtman et al. 1984; Jelev et al. 2001; Lalley 1986) (Fig. 1.2).

1.3. The role of serotonin in the modulation of the eupneic respiratory rhythm

Serotonin is a major neuromodulator in the central nervous system. Raphé 5-HT-producing cells project to respiratory areas in the medulla and pons, including the

reticular formation and phrenic nuclei, and these neurons alter the respiratory rhythm (Hilaire and Duron 1999).

Seventeen 5-HT receptor subtypes have been identified and all but the 5-HT_3 receptor produce cellular effects via G-protein second messenger systems (Fig. 1.3; Kinney 2005; Richter et al. 2003). As such, 5-HT can either hyperpolarize or depolarize neurons, depending on the type of receptor to which it binds and the specific G-protein that is subsequently activated. Several of the receptor subtypes have been implicated in modulation of respiratory rhythm, particulary 5-$HT_{1A,\ 2,\ 4\ and\ 7}$ (Hodges and Richerson 2008).

The bulk of the evidence suggests that 5-HT facilitates the respiratory system. Within the medulla, in both neonatal rats and mice, 5-HT excites respiratory rhythm generation (Hilaire et al. 1997). Intracerebroventricular and *in vitro* administration of agonists increase respiratory frequency (reviewed in Bianchi et al. 1995). The strength of 5-HT facilitation decreases with age throughout the embryonic and post-natal periods (Di Pasquale et al. 1992).

The dominant receptor subtype identified in the 5-HT excitatory modulation of ventilation is the 5-HT_2 receptor. This receptor subtype is found extensively throughout respiratory-related nuclei and DOI (1-[2.5-dimethoxy-4-iodophenyl]-2-aminopropane, a selective 5-$HT_{2A/2C}$ receptor agonist) stimulates breathing in rats *in vivo* and increases hypoglossal nerve output *in vitro* (Al-Zubaidy et al. 1996; Cayetanot et al. 2002). 5-HT_2 receptor activation also depolarizes medullary respiratory neurons (both expiratory and post-inspiratory) in anesthetized cats (Lalley et al. 1995). 5-HT_2 receptors couple with G-proteins that are positively coupled to phospholipase C (PLC), which mediates production of inositol triphosphate (IP_3) and diacylglycerol (DAG) (de Chaffoy et al. 1985). IP_3 leads to calcium release from intracellular stores, and DAG activates protein kinase C (PKC), which inhibits potassium channels leading to membrane depolarization. In addition to respiratory rhythm modulation, activation of 5-HT_{2A} receptors is required for respiratory-related rhythm generation in transverse slice preparations of neonatal mice (Pena and Ramirez 2002). This occurs via endogenous 5-HT acting on 5-HT_{2A} receptors,

which modulate both transient and persistent sodium currents (required for burst generation) in preBötC neurons.

5-$HT_{4/7}$ receptors exert excitatory effects through positive coupling to G-proteins that activate adenylate cyclase thus increasing cyclic adenosine monophosphate (cAMP) levels, in turn activation protein kinase A (PKA) (Manzke et al. 2003). PKA activation directly inhibits voltage-gated potassium channels, resulting in depolarization (Bockaert et al. 2006). 5-HT_4 receptors are abundantly expressed on preBötC neurons, cells involved in the generation of the respiratory-related rhythm (Manzke et al. 2003, 2008; Smith et al. 1991; Wenninger et al. 2004). When the 5-HT_{4A} agonist BIMU8 is added to the perfusate in a perfused *in situ* rat brainstem preparation and injected into a live rat, similar results are obtained. *In situ*, 5-HT_{4A} receptor activation increases phrenic nerve activity and *in vivo* it increases respiratory minute volume (Manzke et al. 2003). The role of 5-HT_7 receptors in breathing is still unclear, though they are also expressed in the preBötC (Richter et al. 2003).

5-HT_{1A} receptors are located pre- and post-synaptically and are coupled to inhibitory G-proteins, causing an inhibition of adenylate cyclase resulting in a decrease of cAMP levels (Richter et al. 2003). This results in decreased neuronal excitability and hyperpolarization through activation of inwardly rectifying potassium channels and inhibition of N and P/Q-type calcium channels (Ladewig et al. 2004). Receptor subtype 1A is the most extensive class of 5-HT receptors in the respiratory network (Richter et al. 2003). An important role for this specific receptor is its autoreceptor function; ligand binding to somatodendritic 5-HT_{1A} receptors inhibits 5-HT release (McCall and Clement 1989; Veasey et al. 1995).

1.4. The hypercapnic ventilatory response

One of the important homeostatic roles of the brain is to regulate arterial blood partial pressure of CO_2 (P_{CO_2}) and pH. This is achieved via a negative feedback loop, with peripheral and central CO_2 chemoreceptors detecting the change in P_{CO_2} and/or pH and eliciting a response through an appropriate change in ventilation that maintains the

levels in a physiological range (pH 7.35-7.45). The brain responds to increases in arterial blood P_{CO_2} by altering ventilatory parameters such as breathing frequency and tidal volume. However, the relative changes in these parameters are variable among species and across developmental stages within a single species (Mortola and Lanthier 1996; Putnam et al. 2005; Strohl et al. 1997). In adult rats, the ventilatory response to 7% inspired CO_2 is an increase in minute ventilation caused by significant increases in both breathing frequency and tidal volume (Taylor et al. 2005). The response to high CO_2 in rats aged 30 days post-natal (P30) is similar to the adult response. A ~250% increase in minute ventilation above eupnea is observed, due to an increase in both tidal volume (~200%) and breathing frequency (~130%) (Davis et al. 2006). This is relevant to my studies as my experimental animals were roughly 30 days of age.

1.5. An overview of central chemosensitivity.

Central chemoreceptors were first identified on the ventrolateral surface of the medulla (Mitchell et al. 1963). Subsequent research has suggested that central chemosensors are located in a diffuse network including but not restricted to cells in the nucleus of the solitary tract (NTS), the locus coeruleus, the midline medullary raphé, the rostral ventrolateral respiratory group (rVRG), the preBötC, the fastigial nucleus, areas just beneath the ventral medullary surface, and the retrotrapezoid nucleus (RTN) (Feldman et al. 2003). Morphological cell types have been identified in at least three sites: glutamatergic cells in the RTN, noradrenergic cells in the locus coeruleus, and serotonergic cells in the medullary raphé (Richerson et al. 2005). The relative contributions of the various CO_2-sensitive sites to the overall breathing response induced by changes in CO_2 are still unknown, and it is unlikely that there is a single population of chemosensitive cells that display an intrinsic response to CO_2 that is similar to the ventilatory response of the animal as a whole (Mitchell 2004). Local CO_2 chemosensitivity may depend on arousal state. Thus it is possible that different chemosensitive nuclei are responsible for altering ventilation depending on whether the animal is asleep or awake (see section 1.7).

1.6. The raphé and serotonergic cells as chemosensors

5-HT-containing neurons were first proposed as chemoreceptors by Richerson and Getting (1987). Growing evidence suggests that 5-HT neurons are likely chemoreceptor candidates, particularly those in the medullary raphé. 5-HT neurons are the single most common neuron type within the raphé (~25%), and 75-90% of these medullary raphé 5-HT neurons are chemosensitive despite the fact that not all of them are respiratory-related (Richerson 2004; Wang et al. 2001). While no conclusive evidence has been found to identify the cellular mechanism responsible for sensing changes in P_{CO_2} in medullary raphé 5-HT cells, pH is most likely detected, and not CO_2 directly (Richerson 2004). Though still not clearly understood, pH is thought to be sensed by a calcium-activated non-selective cation (CAN) current (Richerson 2004; Wu et al. 2009). This current is inhibited by acidosis over a physiological range in pH (7.2 to 7.6) and is permeable to both potassium and sodium (ratio 7:1, respectively). Collectively, the following evidence strongly suggests that 5-HT neurons are chemoreceptors.

1) Single neurons are excited by CO_2 in vitro. In tissue culture, medullary raphé neurons increase their firing rate in response to increasing CO_2, and decrease their firing rate in response to decreasing CO_2 (Wang et al. 1998). The majority of these acidosis-stimulated neurons contain 5-HT (Wang et al. 2001). In addition, these isolated raphé neurons are primarily stimulated by changes in intracellular pH because when CO_2 levels are changed while extracellular pH is kept constant, an increase in firing rate is still observed (Richerson 2004; Wang et al. 2002). These cell culture studies demonstrate that many 5-HT neurons are intrinsically chemosensitive, which is a requirement for a chemosensitive cell.

2) Focal acidification of the raphé stimulates breathing in vivo. Neurons in the raphé alter ventilation in response to direct and indirect changes in pH; however, this response is partially dependent on the arousal state of the animal. Focal acidification of subregions of the medullary raphé increases breathing by 15-20% during sleep in rats while CO_2 microdialysis stimulates ventilation in goats during wakefulness, but not during sleep

(Hodges et al. 2004; Nattie and Li 2001). Microinjection of acetazolamide (which causes tissue acidosis) into the medullary raphé of rats increases ventilation (Bernard et al. 1996).

3) Disruption of 5-HT neurons disrupts ventilatory response to systemic CO_2 in vivo. Inhibition of medullary raphé neurons with muscimol (a $GABA_A$ receptor agonist) reduces the CO_2 response in intact piglets (Messier et al. 2002). Studies using 8-OH-DPAT (8-Hydroxy-*N*,*N*-dipropyl-2-aminotetralin) to inhibit raphé 5-HT neurons (by activating inhibitory $5\text{-}HT_{1A}$ receptors) also show blunting of the CO_2 response. Focal inhibition of 5-HT medullary raphé neurons by 8-OH-DPAT depresses the ventilatory response to inhaled CO_2 in newborn piglets and adult rats (Messier et al. 2004; Taylor et al. 2005). In adult rats *in vivo*, lesions of medullary raphé 5-HT neurons with a 5-HT neuron targeted neurotoxin (saporin conjugated to an antibody to the serotonin transporter, SERT-SAP) dampen the hypercapnic ventilatory response during sleep and wakefulness (Nattie et al. 2004).

4) 5-HT neurons are stimulated in response to inhaled CO_2 in vivo. The only study to date that has published recordings made directly from 5-HT medullary raphé neurons in an awake, unanesthetized animal during a hypercapnic challenge was by Veasey et al. (1995). They found that inhalation of hypercapnic air increases 5-HT neuronal firing rate 160% in cats. Furthermore, hypercapnia induces c-fos expression in the 5-HT neurons of the medullary raphé (Okada et al. 2002; Teppema et al. 1997).

5) 5-HT neurons are closely associated with large arteries. In addition, 5-HT neurons are in close proximity to large medullary arteries, placing them in a functional position to detect blood $P_{CO_2}$and make appropriate changes to maintain pH homeostasis (Bradley et al. 2002). This proximity to large blood vessels would allow them to faithfully sense arterial P_{CO_2}, which would more closely reflect lung ventilation than would the P_{CO_2} of bulk cerebrospinal fluid or of tissues near small capillaries (affected by tissue metabolism).

1.7. The raphé serotonergic system and the hypothalamic hypocretinergic system: implications for arousal state-dependent chemosensitivity

The ventilatory hypercapnic response is greater during wakefulness than sleep. Arousal state affects the ability for several subsets of central chemoreceptors to respond to changes in CO_2, including the raphé (Berthon-Jones and Sullivan 1984; Netick et al. 1984; Phillipson et al. 1977). Also hypercapnia is a potent arousal stimulus during sleep (Berthon-Jones and Sullivan 1984).

1.7.1. Serotonin/raphé chemosensitivity during sleep

The raphé is not homogeneous, and raphé contributions to chemosensitivity differ during various arousal states. While some research shows that particular raphé subregions contribute equally to the ventilatory response to hypercapnia during both sleep and wakefulness (Nattie et al. 2004; Taylor et al. 2005), other data suggest that the contribution of particular raphé areas to chemosensitivity is prevalent during sleep (Nattie and Li 2001). However, methods used in such studies often disrupt sleep, making it difficult to identify the impact of the medullary raphé system in response to hypercapnia in relation to arousal state (Messier et al. 2002). Nonetheless it has been proposed that the raphé serotonergic system may be the dominant chemosensor during sleep (Feldman et al. 2003; Mitchell 2004).

1.7.2. Raphé activity during sleep

Serotonergic neurons maintain wakefulness by suppressing rapid eye movement (REM) sleep through the release of 5-HT (Jacobs and Fornal 1991). The raphé nucleus most commonly correlated with sleep is the dorsal raphé; however, the firing rate of medullary serotonergic neurons also depends on arousal state (Fornal et al. 1985; Heym et al. 1982). Interestingly, serotonergic medullary raphé neurons (in both the raphé pallidus and raphé magnus nuclei) are most active during active wake states, less during slow wave sleep (SWS), and almost quiescent during REM sleep (Fornal et al. 1985; Heym et al. 1982). Whether the arousal state determines the rate of firing of the medullary serotonergic neuron, or whether the release of 5-HT partially contributes to the

arousal state is uncertain. Nonetheless, the relatively constant firing of 5-HT neurons during wakefulness likely provides a tonic facilitation of ventilation, and the decrease in 5-HT neuron activity during sleep may contribute to the decrease in ventilation observed during this arousal state. Sleep removes non-5-HT mechanisms contributing to CO_2 chemosensitivity, unmasking the role of 5-HT neurons in such a response (Dias et al. 2009; Gestreau et al. 2008; Taylor et al. 2005).

1.7.3. Hypocretinergic system

A functional interaction has been found between the serotonergic raphé neurons and hypocretinergic system, which could potentially influence breathing (Brown et al. 2001). The lateral hypothalamus and endogenous release of hypocretin are important contributors to arousal state, in addition to thermoregulation (and shivering thermogenesis) and feeding behavior (Sakurai et al. 1998). Neurons in the lateral hypothalamus synthesize hypocretin-1 (orexin A) and hypocretin-2 (orexin B) (Sakurai et al. 1998). These are neuropeptides derived from the common precursor prepro-orexin. Hypocretins target two G-protein coupled receptors: hypocretin receptor 1 (hcrt-r1) and hypocretin receptor 2 (hcrt-r2) (Fig. 1.4; Sakurai et al. 1998). These receptors have a different affinity for the hypocretin peptides; hcrt-r1 is selective for hypocretin-1 only, while hcrt-r2 nonselectively binds to both hypocretin-1 and -2 (Sakurai et al. 1998). Hcrt-r1 transmits signals through excitatory G-proteins activating PLC. Subsequent activation of IP_3 and PKC results in an influx of extracellular calcium and neuron depolarization (reviewed in Beuckmann and Yanagisawa 2002). Hcrt-r2 is thought to be coupled to either the excitatory or inhibitory G-proteins. The mechanism of the excitatory G-protein-induced depolarization is similar to that of hcrt-r1. The mechanism for hyperpolarization resulting from the inhibitory G-protein is unknown, but is hypothesized to be via potassium efflux (Beuckmann and Yanagisawa 2002).

Hypocretin neurons stimulate arousal and breathing (Ohno and Sakurai 2008; Young et al. 2005). Moreover, defects in the hypocretinergic system are thought to cause narcolepsy in both humans and animals (Chemelli et al. 1999; Peyron et al. 2000; Thannickal et al. 2000).

1.7.4. Effects of hypocretin on ventilation

Anatomical and functional evidence suggests that endogenous hypocretins modulate breathing. Much like the serotonergic system, axons of hypocretin-containing neurons project to respiratory-related nuclei including the NTS, preBötC, the locus coeruleus, hypoglossal and phrenic nuclei (Fung et al., 2001; Hagan et al. 1999; Nakamura et al. 2007; Young et al. 2005). Also, areas corresponding to the preBötC and the phrenic motornuclei are immunoreactive for hcrt-r1 receptors (Young et al. 2005). Data from patients with various respiratory disorders hint at a correlation between breathing and hypocretins. Patients with Guillain-Barre syndrome, a syndrome often associated with respiratory paralysis, have low levels of hypocretin in their cerebrospinal fluid (Ripley et al. 2001). Also, patients with sleep apnea have low blood levels of hypocretin (Sakurai et al. 2005). Experimental evidence also indicates that hypocretins affect ventilation. Injection of hypocretin-1 into the lateral ventricle of a urethane-anesthetized mouse results in a small increase in breathing frequency (~11%) and a larger increase in tidal volume (~75%) (Zhang et al. 2005).

Administration of hypocretin-1 into the preBötC increases diaphragmatic electromyogram activity but does not affect breathing frequency (Young et al. 2005). This is suggestive that hypocretin stimulates respiration primarily through increases in tidal volume (Young et al. 2005). The respiratory phenotype was established in a prepro-orexin knockout mouse (therefore lacking both hypocretin-1 and -2), often used as an animal model for narcolepsy. Frequency and tidal volume show sleep-wake dependency in both wildtype and knockout mice; however, no significant differences in minute ventilation are observed between the two phenotypes (Nakamura et al. 2007).

1.7.5. Link between hypocretin and 5-HT raphé neurons

Much of the network linking hypocretin and the raphé is described in relation to the dorsal raphé. Hypocretin-1 excites 5-HT dorsal raphé neurons both in slice and *in vivo* (Brown et al. 2001; Takahashi et al. 2005). In addition, hcrt-r1 and 5-HT co-localize in the dorsal raphé, and polymerase chain reaction (PCR) studies show expression of both hypocretin receptors in 5-HT neurons in this area (Brown et al. 2002; Wang et al. 2005).

These hypocretinergic axons synapse directly with 5-HT dendrites in the dorsal raphé (Nambu et al. 1999; Wang et al. 2005). Although most evidence linking hypocretin and 5-HT neurons is based on studies of the dorsal raphé, hypocretinergic receptors are found in the medullary raphé as well. Low density expression of both hcrt-r1 and hcrt-r2 is found in the raphé magnus, while a moderate density of hcrt-r1 with no expression of hcrt-r2 is found in the raphé obscurus, (Marcus et al. 2001). Also, staining shows hypocretin axon terminals in the raphé magnus and pallidus (Nambu et al. 1999). A much denser innervation of hypocretin-1 fibers to raphé pallidus is observed (Zheng et al. 2005). There are also reciprocal connections from the raphé to the lateral hypothalamus, though the 5-HT nerve endings terminating there mainly arise from the dorsal and median raphé nuclei (Muraki et al. 2004). 5-HT directly hyperpolarizes hypocretin neurons via 5-HT_{1A} receptors (Muraki et al. 2004).

1.7.6. Link between hypocretin and hypercapnia

Several studies have shown correlations between the hypocretinergic system and hypercapnia. Hypocretin neurons themselves appear to be chemosensitive. Both CO_2 and H^+ stimulate hypocretin neurons via inhibition of post-synaptic leak-like K^+ channels; however, the degree of hypercapnia needed to produce a response is relatively high compared to the sensitivity of other neuron types (such as 5-HT neurons) (Williams et al. 2007). Also, Nakamura et al. (2007) illustrate a relationship between the effect of hypocretins on the response to hypercapnia and arousal state, using a knockout mouse lacking hypocretin-1 and -2 and a wildtype mouse, and determining sensitivity to a mild (5%) and severe (10%) hypercapnic challenge. These challenges increase ventilation in both genotypes. The wildtype shows a state-dependent sensitivity; these mice are more responsive when awake than during SWS or REM (Nakamura et al. 2007). However, the knockout shows a similar response to CO_2 no matter the arousal state. No difference is observed in responses to hypercapnia between wildtype and knockout during sleep states. However, the CO_2 responses during wakefulness are blunted in the knockout compared to the wildtype (Nakamura et al. 2007). This would suggest that hypocretins contribute to the increased hypercapnic sensitivity during the awake state. This complements the

observation that hypocretin neurons fire spontaneously during wakefulness periods and less, or not at all, during sleep (Estabrooke et al., 2001; Lee et al. 2005). Given the anatomical and physiological evidence linking the hypocretinergic and serotonergic systems, and the involvement of both of these systems in arousal, they may interact to promote a response to hypercapnia.

1.8. The perfused *in situ* juvenile rat brainstem preparation

This preparation has been widely used in recent years and is an appropriate model for neural control of ventilation studies primarily because it maintains the complexity of respiratory networks (Fig. 1.5; Harris and St-John 2003; Harris et al. 2003; Paton 1996; St-John and Paton 2000; Wilson et al. 2001). The preparation is decerebrate; however, the balance of the central nervous system is intact, including the brainstem containing the entire respiratory network. As such, the *in situ* preparation maintains similar control of the ventilatory system to that found *in vivo*. A comparable response to hypercapnia is observed both *in vivo* and *in situ*, where phrenic activity increases both in burst frequency and peak amplitude with increased CO_2 (St-John and Paton 2000). The phrenic burst shapes for eupnea and gasping are similar when measured in this *in situ* preparation and *in vivo* (Paton 1996; St-John and Paton 2000). In addition, the preparation receives adequate tissue oxygenation (Wilson et al. 2001), while more isolated brainstem preparations such as the en bloc preparation do not.

When compared to animals *in vivo*, this preparation has added benefit in that it is not influenced by anesthesia beyond the initial dissection. Also, it allows for easy administration of pharmacological agents via addition to the perfusate and equilibration with tissues through normal micro-circulation. One criticism is that the preparation must be kept hypothermic at 31°C to enhance its viability (Paton 1996). This results in a slower phrenic burst frequency when compared to frequencies of rats *in vivo* at euthermia (37°C). However, this slower breathing frequency is similar to the depression observed in hypothermic rats *in vivo* (Torbati et al. 2000). With the above taken into consideration,

the perfused *in situ* juvenile rat brainstem preparation is an appropriate model to determine the role of 5-HT in central chemosensitivity.

1.9. Use of a genetic model

Advancement of molecular tools has made available animal models in which specific neurotransmitter systems have been genetically altered. Our model for studying the role of 5-HT neurons in ventilatory control is the *Lmx1b*$^{f/f/p}$ transgenic mouse. These mice exhibit knockout of the transcription factor *Lmx1b* selectively in central nervous system cells that express Pet-1, a transcription factor associated with differentiation of serotonergic neurons. This results in a near-complete (>99%) and specific absence of central serotonergic neurons as well as a coincident marked depletion of central 5-HT (Ding et al. 2003; Zhao et al. 2006). This *Lmx1b*$^{f/f/p}$ mouse is an invaluable tool as it enables us to focus more specifically on the role of serotonergic versus non-serotonergic neurons in central chemosensitivity. As adults, *Lmx1b* knockouts breathe normally at rest but display a blunted ventilatory response to hypercapnia (about 50% less than wildtype mice) and abnormal thermoregulatory responses (Hodges et al. 2008).

1.10. Ventilatory chemosensitivity and Sudden Infant Death Syndrome (SIDS)

The role of the raphé in homeostatic reflexes, and particularly 5-HT neuron contributions to central chemoreception, hold significance in relation to the recent hypothesis that a subset of SIDS cases result from dysfunction in the medullary serotonergic system (Kinney et al. 2009; Nattie 2009). This dysfunction is proposed to compromise infant reflex responses to exogenous stressors (such as heat, high CO_2, and low O_2). However, the primary evidence linking SIDS with abnormalities in the medullary serotonergic system is anatomical, derived from radioligand binding studies.

1.10.1. SIDS triple-risk model

SIDS is defined as "the death of an infant under one year of age that remains unexplained after a complete clinical review, autopsy, and death scene investigation" (Kinney et al. 2009; Nattie 2009). It is a leading cause of infant mortality, with an overall

incidence of 0.6 per 1000 births (Kinney 2005). The occurrence of SIDS has been described by a triple-risk model (Figure 1.6; Filiano and Kinney 1994). This model proposes that SIDS is likely to occur when three factors are coincident: 1) an underlying vulnerability, 2) an exogenous stressor, and 3) a critical developmental time period for homeostatic control.

In the SIDS triple-risk model, the "critical developmental period" is presumed to be the period of post-natal development, a time of change in homeostatic control (Kinney et al. 2001). The greatest risk period is during the first 6 post-natal months, with a peak risk at 2-4 months (Kinney 2005).

The underlying vulnerability is a physiological condition that impairs the infant's response to life-threatening situations imposed by the exogenous stressors. The exogenous stressors are commonly occurring but potentially life threatening circumstances such as an increase in P_{CO_2}. These circumstances are normally overcome by critical homeostatic reflexes such as the hypercapnic ventilatory response. The model proposes that, in a vulnerable infant, reflex responses are compromised such that exogenous stressors can not be overcome, resulting in SIDS (Filiano and Kinney 1994). Much current research is devoted to identifying factors contributing to "underlying vulnerability" to SIDS. One factor is hypothesized to be malfunction in the brainstem serotonergic system.

1.10.2. The serotonin malfunction hypothesis for SIDS

The serotonin malfunction hypothesis was proposed based on increasing anatomical evidence of abnormalities in the medullary 5-HT system in SIDS cases. The medullary 5-HT system is abnormal in at least 50% of SIDS cases (Kinney 2005). In two separate datasets of SIDS cases, Kinney et al. (2001, 2003) measured 5-HT binding density using quantitative autoradiography with the nonselective radioligand ^{3}H-LSD, which binds to 5-HT_{1A-D} and 5-HT_2 receptors. They found that in both datasets, 5-HT receptor binding was significantly lower in the arcuate nucleus and the raphé obscurus in SIDS cases when compared to age-matched controls. This is particularly interesting due to evidence of a relationship between the arcuate nucleus and the raphé nuclei to

chemosensitivity (Paterson et al. 2006a; Richerson 2004). Although the Kinney et al. (2001, 2003) studies are important for their demonstration of altered receptor binding density, they do not specify which receptor subtypes are involved. In a more recent study, Paterson et al. (2006b) looked at the 5-HT neuron count and density, 5-HT_{1A} receptor binding density, and 5-HTT (the 5-HT reuptake transporter that controls the amount of 5-HT in the synaptic cleft) binding density in SIDS cases compared to age-matched controls. The 5-HT neuron count and density are significantly higher in SIDS cases in regions of the midline raphé, lateral extraraphé, and ventrolateral medullary surface; however, many of these neurons are immature (non-functional). 5-HT_{1A} receptor binding is significantly lower in SIDS cases in all nuclei tested, excluding the inferior olive. No differences are seen in 5-HTT binding density. The ratio of 5-HTT to the 5-HT neuron count is lower in the raphé obscurus, indicating a relative reduction of 5-HTT expression per 5-HT neuron. The above abnormalities, however, do not indicate if there is an excess or a deficit of available 5-HT in SIDS cases. What remains to be determined is the level of available 5-HT in normal versus SIDS brainstems. In general, dysfunction in normal homeostatic regulation via 5-HT is presumed to contribute to SIDS vulnerability.

1.11. Relevance of dissertation research to an understanding of SIDS

It is not the intent of this dissertation to use the perfused *in situ* juvenile rat preparation as a model for SIDS. I aim to identify the functional consequences of pharmacologically induced serotonergic dysfunction in this animal model system. These data will identify if experimentally induced 5-HT system dysfunction can compromise critical homeostatic reflexes which, if occurring in an infant, would enhance vulnerability to SIDS. My studies are relevant in that they provide further physiological evidence to support the hypothesis that a subset of SIDS cases is due to abnormalities in the medullary serotonergic system, which may prevent vulnerable infants from responding appropriately to exogenous stressors such as high CO_2.

1.12. Aims

The experimental focus of this dissertation is to provide further physiological evidence for the link between serotonin malfunction and faults in CO_2 chemosensitivity. CO_2 may be a key exogenous stressor in SIDS cases. Since SIDS occurs primarily during sleep, I also focus on the potential functional interaction between the serotonergic and hypocretinergic systems, as hypocretins are known to play a role in arousal and potentially in chemosensitivity. I achieve much of this focus by using an unanesthetized *in situ* perfused juvenile rat brainstem preparation. This preparation is innovative in that previous raphé chemosensitivity studies have been performed using either anesthetized animals or reduced preparations such as *in vitro* brainstem preparations or cell cultures, which do not maintain the connectivity of respiratory networks. The following experiments were designed to address specific aims that support the overarching hypothesis: **the serotonergic and hypocretinergic systems are involved in modulating ventilation and hypercapnic ventilatory responses.**

Specific Aim I. To identify that 5-HT neurons and specific 5-HT receptor subtypes are involved in the hypercapnic neuroventilatory response *in situ*.

I test the hypothesis that the 5-HT system is involved in modulating the neuroventilatory rhythm in response to hypercapnia. Using the unanesthetized *in situ* perfused juvenile rat brainstem preparation (from here on referred to as the *in situ* preparation), I first define the changes in neuroventilation caused by altering levels of CO_2 in the perfusate. This is achieved by recording phrenic nerve burst parameters (frequency and amplitude) and using them as neural correlates for ventilatory parameters (breathing frequency and tidal volume). I then identify the importance of 5-HT neurons, and 5-HT_1 and 5-HT_2 receptors in neuroventilatory responses to hypercapnia, by pharmacological alteration of the 5-HT system during a hypercapnic challenge. I expect that disruption of the 5-HT system will alter neuroventilation and dampen the neuroventilatory hypercapnic response. Thus these studies will illustrate the contribution of the serotonergic system in neuroventilation and chemosensitivity. (Chapter 2)

Specific Aim II. To identify the role of the hypocretinergic system in neuroventilation and the hypercapnic neuroventilatory response. I test the hypothesis that hypocretins contribute to neuroventilation and promote the hypercapnic neuroventilatory response. Using the *in situ* preparation, I identify the effects of hypocretin-1 administration and of hcrt-r1 antagonism on phrenic neuroventilation, and changes in neuroventilation with hypercapnia (chemosensitivity). I also identify the effects of hypocretin-1 on the hypoglossal nerve output in a neonatal rat rhythmic medullary slice preparation. I predict that addition of hypocretin-1 will stimulate neuroventilation and enhance chemosensitivity. Conversely, antagonism of hcrt-r1 is expected to dampen neuroventilation and blunt chemosensitivity. Thus these studies will illustrate the contribution of the hypocretinergic system to neuroventilation and chemosensitivity. (Chapter 3)

Specific Aim III. To identify the role of serotonin neurons and of hypocretinergic modulation in the neuroventilatory response to acidosis. The objectives in this Specific Aim are two fold. The first sub-aim is to establish that serotonergic neurons are important in central sensitivity to pH. I test the hypothesis that serotonin neurons contribute to the neuroventilatory response to acidosis in an *in vitro* rhythmic medullary slice preparation. I determine the response of hypoglossal nerve output to changes in pH in a medullary slice preparation derived from a wildtype and $Lmx1b^{f/f/p}$ mouse (which lacks central serotonergic neurons). I expect that responses in slices lacking central serotonergic neurons will be blunted compared to responses in wildtype slices.

The second sub-aim is to establish that hypocretinergic modulation of neuroventilation and chemosensitivity is determined by an impact of hypocretin on 5-HT neurons. I test the hypothesis that hypocretins contribute to neuroventilation and promote the neuroventilatory response to acidosis through serotonergic neurons. In rhythmic medullary slices derived from wildtype and $Lmx1b^{f/f/p}$ mice, I identify whether changes in hypoglossal nerve activity generated by hypocretin-1 and hypocretin receptor antagonists depend on the presence of 5-HT neurons. I also identify the effects of hypocretin receptor antagonism on hypoglossal nerve responses to acidosis and whether or not this is

dependent on the presence of 5-HT neurons. I expect that responses in slices lacking 5-HT neurons will be blunted compared to wildtype slices. Thus these studies will illustrate the 5-HT neuron-dependence of hypocretinergic impacts on neuroventilation and chemosensitivity (Chapter 4).

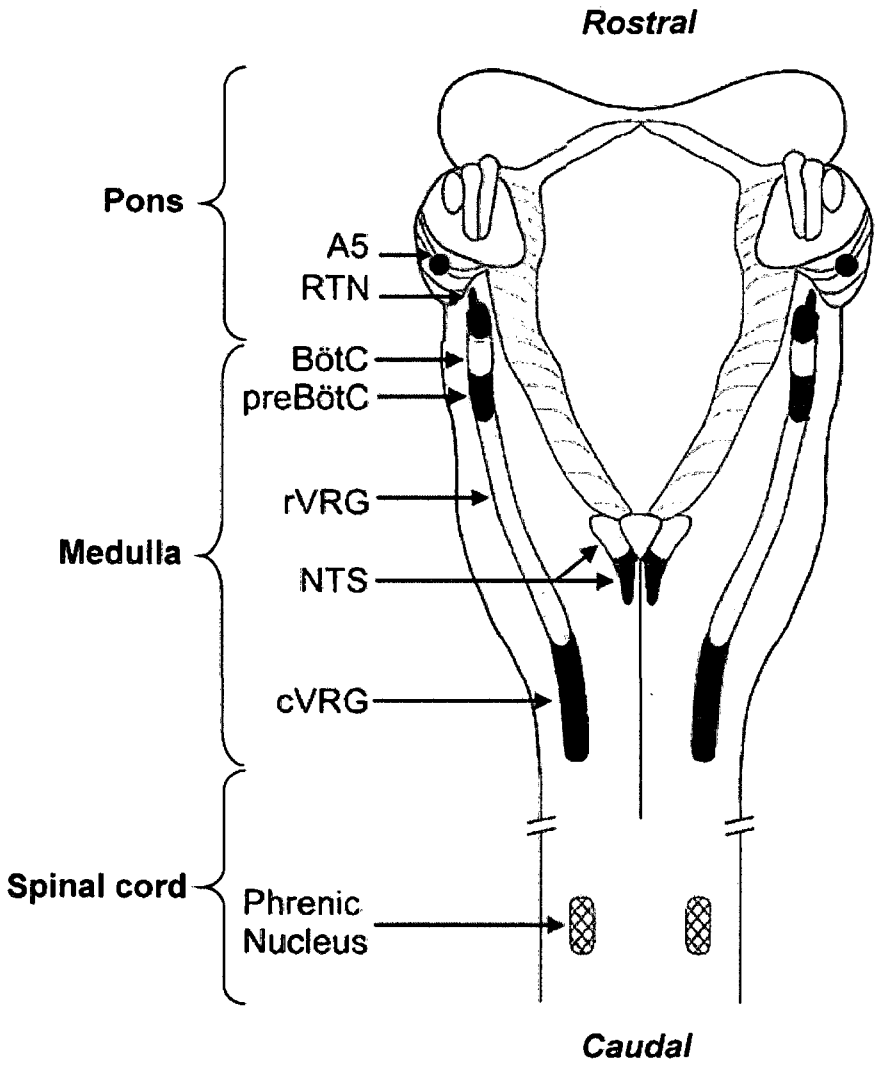

Figure 1.1 Dorsal view of rat brainstem and cervical spinal cord indicating the regions important in the generation of breathing. Abbreviations include: A5, pontine A5 group; RTN, retrotrapezoid nucleus; BötC, Bötzinger complex; preBötC, preBötzinger complex; rVRG, rostral ventral respiratory group; NTS, nucleus tractus solitarius; cVRG, caudal ventral respiratory group. Modified from Rekling and Feldman 1998.

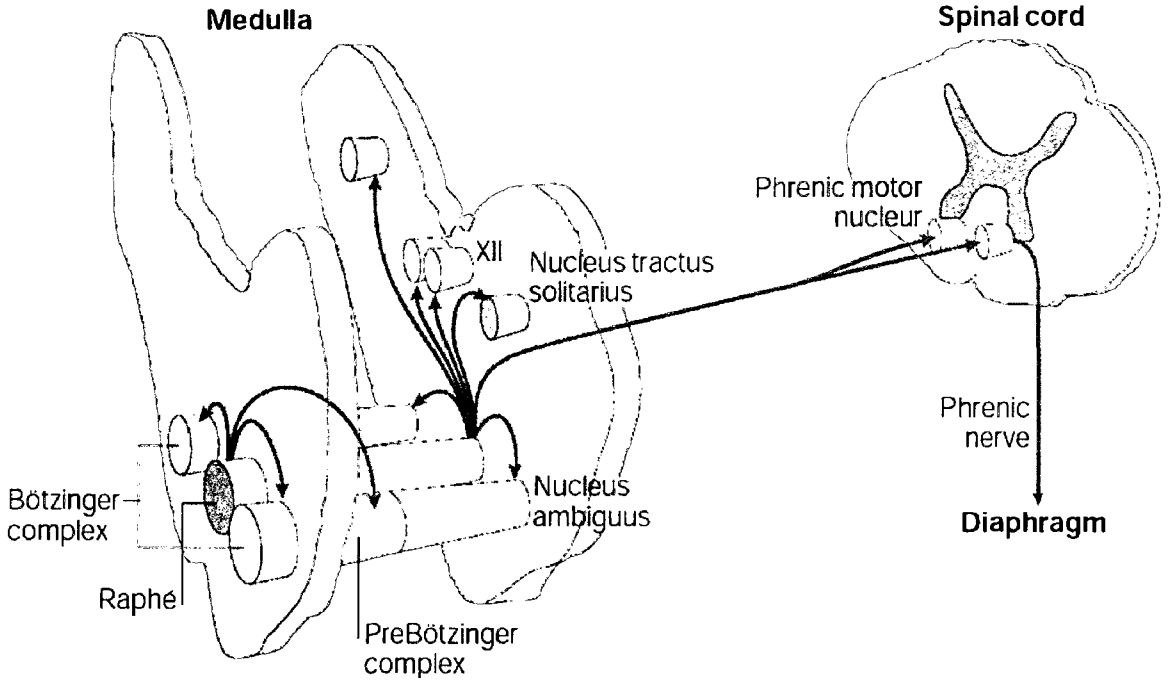

Figure 1.2 Schematic of the anatomical connections between the medullary raphé, and respiratory-related nuclei and motonuclei. Abbreviation XII indicates hypoglossal motonuclei. From Richerson 2004.

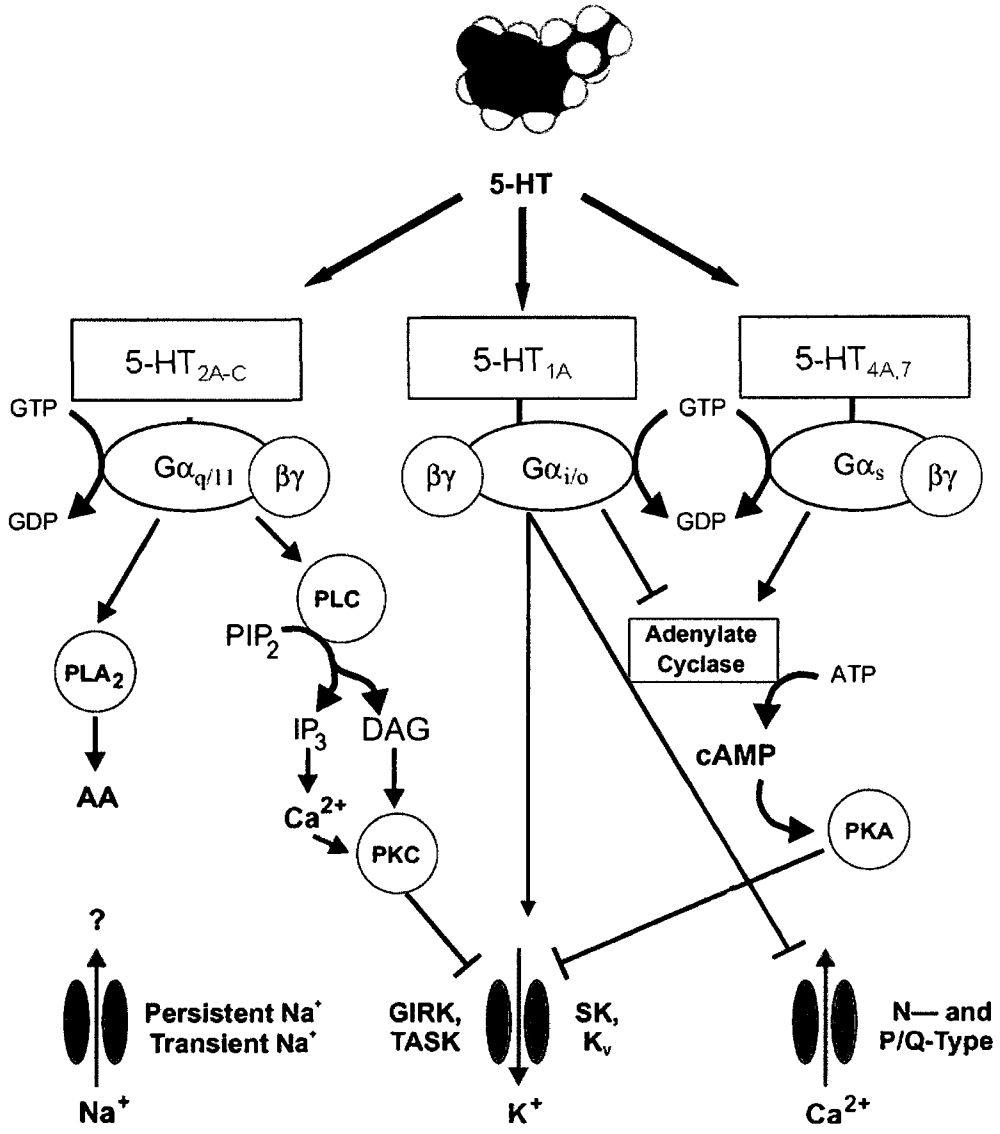

Figure 1.3 Schematic of cellular pathways activated by 5-HT receptor subtypes associated with breathing. Shown are some of the known (arrow= activation, blunt line = inhibition) and postulated (question marks) pathways and effectors through which 5-HT affects membrane excitability. Abbreviations include: AA, arachidonic acid; DAG, diacylglycerol; GIRK, G-protein-gated inwardly rectifying potassium channel; IP_3, inositol trisphosphate; *K*V, voltage-gated potassium channel; PIP_2 phosphatidylinositol bisphosphate; PK, protein kinase; PL, phospholipase; SK, small conductance calcium-activated potassium channel; TASK, TWIK-related acid-sensitive potassium channel. Modified from Hodges and Richerson 2008.

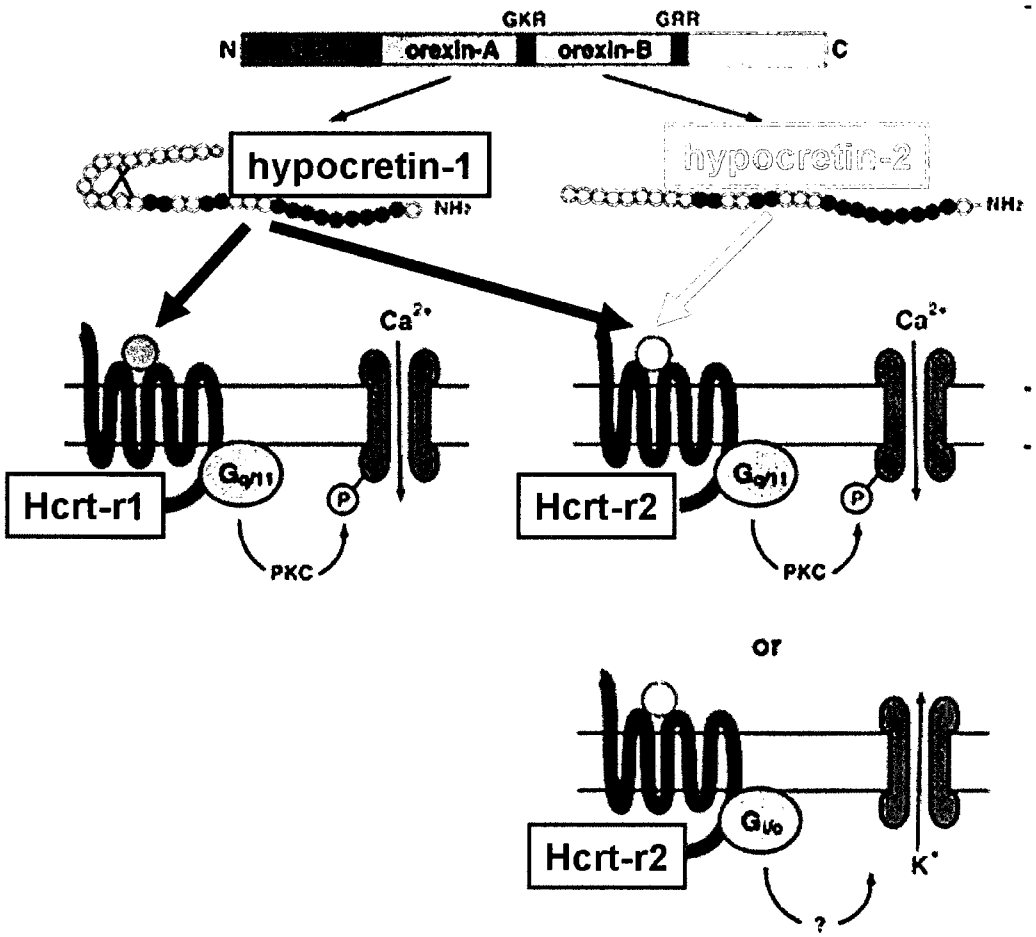

Figure 1.4 Schematic of hypocretinergic signaling. Neuropeptides hypocretin-1 and -2 are derived from a common precursor gene. Hypocretin-1 can bind with both hypocretin receptor types (hcrt-r1 and hcrt-r2) while hypocretin-2 has high affinity only for hcrt-r2. Modified from Beuckmann and Yanagisawa 2002.

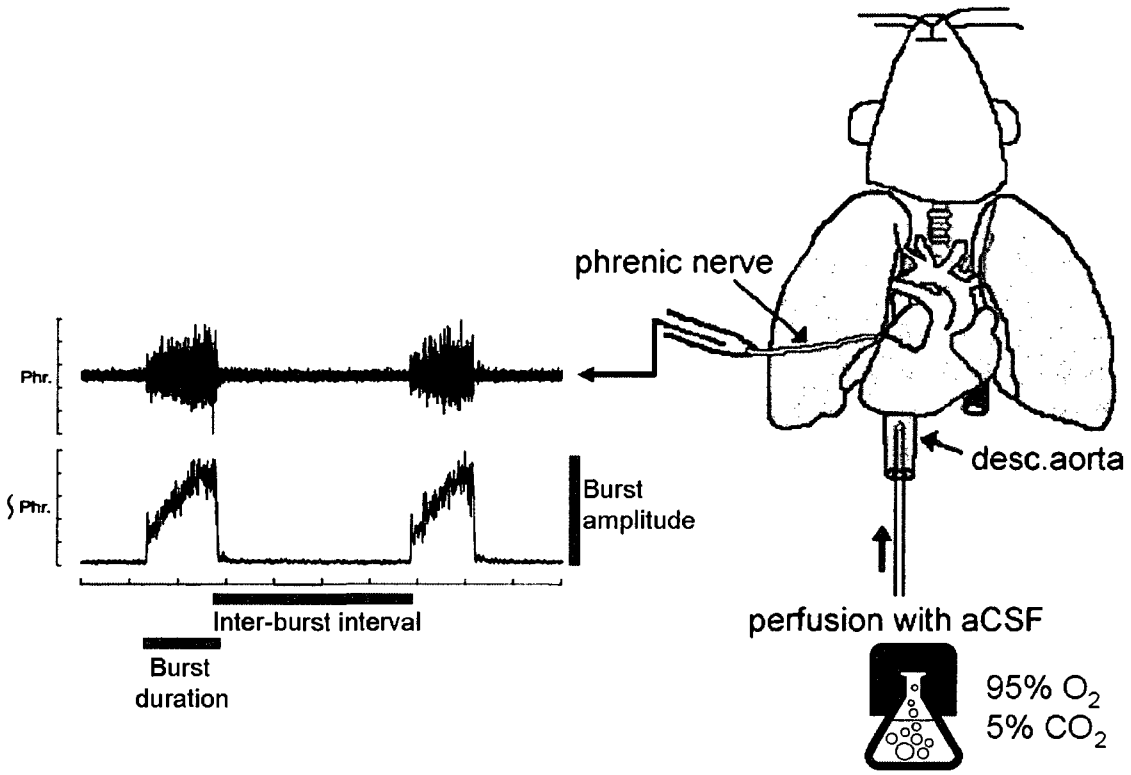

Figure 1.5 Schematic of the perfused *in situ* juvenile rat brainstem preparation. Artificial cerebrospinal fluid (aCSF) is equilibrated with a gas mixture (95% O_2/ 5% CO_2 during control conditions) and pumped by a roller pump through a bubble trap and filter before entering the descending aorta via a double-lumen catheter. Phrenic nerve activity is recorded via a glass suction electrode and monitored by a computer data acquisition system. Phrenic nerve burst parameters are characterized as burst duration, inter-burst interval (period between the bursts), burst amplitude, and burst frequency. Included is sample of a raw trace (top) and integrated trace (bottom) of phrenic nerve output.

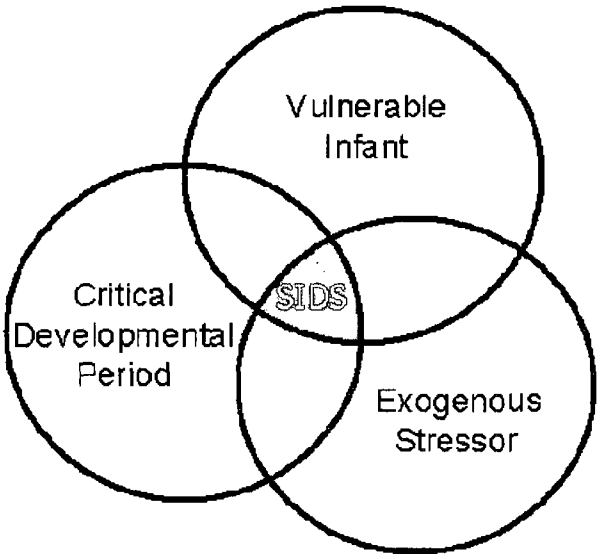

Figure 1.6 The triple-risk model proposes that an infant may die of SIDS if 3 factors occur simultaneously: 1) an underlying vulnerability, 2) an exogenous stressor, and 3) a critical developmental time period for homeostatic control. Adapted from Kinney et al. 2001.

1.13. References

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Chapter 2 Serotonergic mechanisms are critical for central neuroventilatory chemosensitivity *in situ*[1]

Abstract

Studies *in vitro* suggest that serotonin-synthesizing neurons (5-HT neurons) may be important for CO_2 chemosensitivity. The roles of these neurons in modulating breathing *in vivo*, however, are less clear. We used a perfused *in situ* juvenile rat brainstem preparation that exhibits patterns of phrenic nerve discharge akin to breathing *in vivo*. Exposure of this preparation to hypercapnia increased phrenic burst frequency and/or amplitude, neural correlates of hypercapnic ventilatory responses in breathing frequency and tidal volume *in vivo*. This study was designed to assess the importance of 5-HT neurons and 5-HT receptor activation in modulating frequency and amplitude components of the hypercapnic neuroventilatory response. Neuroventilatory responses to hypercapnia were assessed without and with application of methysergide (5-10 μM, a mixed $5\text{-HT}_{1/2}$ receptor antagonist), 8-OH-DPAT (1.5 μM, an agonist of 5-HT_{1A}), and ketanserin (5 μM, a 5-HT_2 antagonist). All pharmacological manipulations abolished hypercapnic responses. Our data illustrate that 5-HT neurons and 5-HT receptor activation are critical for CO_2 responses *in situ*, and support a hypothesis that such neurons play an important role in central ventilatory chemosensitivity *in vivo*.

2.1. Introduction

Brainstem serotonin-synthesizing neurons (5-HT neurons) project widely throughout the central nervous system, and comprise a complex and broad neuromodulatory system (Jacobs and Azmitia 1992). 5-HT neurons have been implicated in the modulation of

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brainstem respiratory neurons, influencing breathing in a number of ways, ranging from facilitation and/or stabilization of the respiratory-related rhythmogenesis *in vitro* (Pena and Ramirez 2002; Ptak et al. 2009; Richter et al. 2003) to the generation and/or stabilization of gasping (Toppin et al. 2007; Tryba et al. 2006). Substance P (SP) and thyrotropin-releasing hormone (TRH) both co-localize in many medullary 5-HT neurons, suggesting that these neurons may act via a range of post-synaptic mechanisms that could involve neurotransmission via a combination of 5-HT, SP, and/or TRH (Holtman et al. 1984).

Much evidence shows that 5-HT neurons are critically involved in the brain's ability to effectively detect changes in P_{CO_2}/pH and elicit ventilatory responses that return arterial blood gases to a homeostatic level (see Corcoran et al. 2009a; Richerson 2004 for review). The medullary raphé, composed in large part of 5-HT neurons, has been identified as one of the primary sites for central chemosensitivity (Bernard et al. 1996; Dias et al. 2007; Hodges et al. 2004; Messier et al. 2002; Nattie and Li 2001; Nattie et al. 2004; Penatti et al. 2006; Taylor et al. 2005; Veasey et al. 1995). 5-HT mechanisms are linked to central chemosensitivity, as treatments with selective 5-HT reuptake inhibitors augment ventilatory responses to hypercapnia in intact animals, whereas pharmacologic inhibition or neurotoxic lesions within specific subsets of 5-HT neurons decrease such responses (Nattie et al. 2004; Taylor et al. 2004, 2005). More recently, genetically modified mice illustrate that 5-HT neurons contribute to hypercapnic ventilatory responses. Adult male Pet-$1^{-/-}$ mice, which lack 70% of central 5-HT neurons, display a blunted CO_2 response (Hodges et al. 2005). Also, mice exhibiting a more extreme phenotype in which the transcription factor *Lmx1b* has been selectively knocked out in Pet-1-expressing cells (*Lmx1b*$^{f/f/p}$; resulting in >99% loss of central 5-HT neurons) have a 50% reduction in the hypercapnic ventilatory response compared to wildtype (Hodges et al. 2008). A partial deficit in CO_2 chemosensitivity in animals lacking some or all 5-HT neurons implicates 5-HT neuron-mediated mechanisms in such chemosensitivity *in vivo*. It is not clear if the residual chemosensitivity represents 5-HT neuron-independent chemosensitive mechanisms that normally contribute to ventilatory

responses, or if it results from compensatory mechanisms emergent in genetically modified experimental systems.

Hypercapnic acidosis increases the firing rate of 5-HT neurons in rat primary cell culture and acute brainstem slices (Richerson 1995; Wang et al. 2001). A subset of 5-HT neurons function as respiratory chemoreceptors and alter ventilation to maintain blood gas/pH homeostasis (reviewed by Richerson 2004). This proposed role of 5-HT neurons, and the neurotransmitter and receptor mechanisms involved, however, remain controversial (Richerson et al. 2005; Guyenet et al. 2005).

Much of the research supporting a critical role of 5-HT neurons in ventilatory chemosensitivity in rodents has been performed in primary cell culture and acute brainstem slices (Wang et al. 1998, 2001, 2002). Criticism of these studies suggests that 5-HT neuron chemosensitivity *in vitro* may not reflect chemosensitive mechanisms functioning *in vivo*. The latter opinion, however, is primarily based on studies conducted in anesthetized *in vivo* preparations, without consideration of the potential confounding influences of anesthesia, the age of animals tested, or the specific location of 5-HT neurons under investigation (see Corcoran et al. 2009a for review). The experimental system used in our current study, the unanesthetized, decerebrate arterially perfused *in situ* juvenile rat brainstem preparation (Paton 1996), retains the complexity of intact respiratory networks *in vivo*, while avoiding potentially confounding influences of anesthesia or emergent properties of genetically modified model organisms. Numerous studies have validated that this perfused *in situ* brainstem preparation exhibits neuroventilatory activity patterns representative of automatic ventilation comparable to eupnea *in vivo*, and that this preparation retains sensitivity to hypercapnic acidosis (Day and Wilson 2005; St-John and Paton 2000; Toppin et al. 2007). In the present investigation, we used the perfused *in situ* brainstem preparation to assess the importance of 5-HT neuromodulation in altering respiratory drive in response to hypercapnia. The aim of this study was to evaluate the hypothesis that 5-HT neurons and 5-HT neuromodulation are critical in the hypercapnic neuroventilatory responses in our relatively intact preparation.

2.2. Methods

2.2.1. Experimental preparations

Animal husbandry and experimental procedures conformed to guidelines set by the National Institutes of Health Office of Laboratory Animal Welfare and the United States Department of Agriculture Animal Welfare Act, and were approved by an Institutional Animal Care and Use Committee of the University of Alaska Fairbanks. Juvenile male albino rats (24-30 days old; 70-140 g; Sprague-Dawley strain; Charles River and Simonson Laboratories) were used to derive decerebrate arterially perfused *in situ* brainstem preparations, as previously described (St-John and Paton 2000; Toppin et al. 2007). This general method varied slightly depending on the experimental series; differences are indicated where applicable. Briefly, animals were pretreated with intraperitoneal heparin sodium injection (0.7 – 1.5 mls, 1000 USP; Baxter, Deerfield, IL) to prevent blood clotting during subsequent surgery. Procedures were preceded by deep anesthesia using halothane in experimental series I and isofluorane in series II and III. Anesthesia was assessed by an absence of withdrawal response to firm toe pinch and cessation of spontaneous breathing movements. Anesthesia was discontinued as animals were decerebrated rostral to the superior colliculi, and subsequent procedures were conducted in the absence of anesthesia. Preparations were bisected below the diaphragm and immersed in ice-chilled saline. Preparations were then placed, supine (series I and II) or prone (series III), in a recording chamber. The descending aorta was cannulated with a double-lumen catheter and preparations perfused with 31°C solution. The perfusate contained the following in distilled water: $MgSO_4$ (1.0 mM); KH_2PO_4 (0.25 mM); KCl (4.0 mM); $NaHCO_3$ (25 mM); NaCl (115 mM); $CaCl_2$ (2.0 mM); dextrose (10 mM) and Ficoll 70 (0.1785 mM). Under baseline conditions, the perfusate was equilibrated with 95% O_2-5% CO_2 (perfusate pH ~7.4). Perfusion pressure was maintained at either approximately 80 mmHg (series I), or 40-60 mmHg (series II and III) by adjustments in flow rate achieved through regulating the speed of a peristaltic perfusion pump. The initial 50 ml of perfusate circulated through each preparation was discarded to eliminate residual blood, and the remaining volume (approximately 300 ml) was collected and

recirculated. The neuro-muscular blocker gallamine triethidodide (60 mg/L) was added to the perfusate to eliminate spontaneous respiratory movements. One phrenic nerve was isolated from the diaphragm and drawn into a glass suction electrode to record efferent nerve activity (phrenic neurogram). The signal was amplified (×10,000; DAM50, WPI) and filtered (band-pass 10 Hz – 1 kHz). In series I, data were digitized at 2 kHz and archived with a computer data acquisition system (DataPac, Advance Instrument). Digitized data were full-wave rectified and averaged over 50 msec intervals. In series II and III, phrenic neurogram data were digitized at 2 kHz (ADInstruments, Powerlab), directly and again on a second channel after being passed through a moving averager (CWE; 50 msec time constant).

2.2.2. Treatments: pharmacological agents and hypercapnia

We altered 5-HT neuron activity or 5-HT neuromodulation by administering agonists and antagonists of the 5-HT_1 and/or 5-HT_2 class of receptors in three experimental series. As a mixed 5-HT_1 and 5-HT_2 receptor antagonist, methysergide maleate was used in concentrations (5-10 μM; series I). The 5-HT_{1A} receptor agonist (*R*)-(+)-8-hydroxy-2(di-*n*-propylamino) tetralin (8-OH-DPAT; 1.5 μM) was administered in series II. Populations of 5-HT_{1A} receptors are located on serotonin-releasing neurons and act as autoreceptors. Activation of 5-HT_{1A} autoreceptors through 8-OH-DPAT (or endogenous extracellular serotonin) results in hyperpolarization, decreased 5-HT neuron firing, and reduced neurotransmitter release (McCall and Clement et al. 1989; Sharp et al. 1989). 8-OH-DPAT is commonly used in respiratory studies to alter 5-HT neuron transmitter release (Messier et al. 2004, Taylor et al. 2005). Finally, in the third experimental series, we administered the high affinity 5-HT_2 class receptor antagonist ketanserin tartrate (ketanserin; 5 μM). All drugs were obtained from Sigma Aldrich. Drug doses were chosen that saturate targeted receptor pools (higher than published Ki values in other systems; Herndon et al. 1992; Marazziti et al. 1997; Newman-Tancredi et al. 1998) and that were lower than those shown to obscure eupneic burst pattern in our preparation (Toppin et al. 2007).

The level of CO_2 bubbled in the perfusate to administer hypercapnia varied between the experimental series and was chosen to elicit biologically relevant changes in neuroventilation. In all series baseline CO_2 was 5% (normocapnia). In series I, 7% CO_2 was chosen for the hypercapnic challenge, while 9% was used in series II and III. A subset of pilot experiments exhibited abnormally high baseline breathing frequencies and paradoxical ventilatory responses to hypercapnia. As consistent ventilatory responses were obtained in the majority of preparations exhibiting baseline frequencies below 50 bursts/minute, preparations exhibiting baseline frequencies greater than 50 bursts/minute were excluded from present analysis. The range of frequencies exhibited by preparations included in our studies match those commonly reported by other researchers using this preparation.

2.2.3. Experimental protocols

Series I: methysergide

Phrenic nerve activity was monitored for roughly 40 minutes under control conditions, and for 10 minutes subsequent to a switch to a hypercapnic perfusate. Phrenic burst parameters during the last 5 minutes of the CO_2 application were analyzed and compared to the 5 minutes prior to the hypercapnic challenge, to determine hypercapnic sensitivity. This hypercapnic exposure protocol was conducted, either in the absence of pharmocological manipulation (sham treatment; $n = 4$) or when methysergide was present in both control and hypercapnic perfusates at either 5 ($n = 4$) or 10 ($n = 4$) μM.

Series II: 8-OH-DPAT

During this series, phrenic burst frequency tended to increase over time (also reported by Day and Wilson (2005), although not observed in Series I) but stabilized after approximately 60 minutes. Neuroventilatory activity was monitored for 60 – 75 minutes to assure a stable baseline. In pilot and control experiments, repeated CO_2 exposures produced consistent and repeatable increases in phrenic burst frequency with hypercapnia in a given preparation (data not shown). As such, a repeated measures design was employed, with all 6 animals undergoing a hypercapnic challenge both before and

following 8-OH-DPAT application. Preliminary experiments revealed that 5 minutes was sufficient to reach the maximal neuroventilatory response to this level of hypercapnia.

After baseline measurements, the preparation was exposed to hypercapnia via the perfusate and then returned to normocapnia for an additional 5 minutes. A final concentration of 1.5 μM 8-OH-DPAT was applied during exposure to normocapnia. After 5 minutes, the hypercapnic challenge was repeated under the continued influence of 8-OH-DPAT. Phrenic activity during the last minute of normocapnia, hypercapnia, normocapnia, normocapnia with 8-OH-DPAT, and hypercapnia with 8-OH-DPAT were analyzed.

Series III: ketanserin

As with the second series, preparations were subjected to an equilibration period of ~ 60 minutes to achieve stable baseline phrenic burst activities. This experimental series also utilized a repeated measures design ($n = 8$). After a stable baseline was reached, preparations were exposed to 5 minutes of hypercapnia followed by 5 minutes of normocapnia. Ketanserin was added to the perfusate (5 μM) and, following 5 minutes of normocapnic ketanserin exposure, the hypercapnic challenge was repeated. Phrenic activities of the final minute of normocapnia, hypercapnia, normocapnia, normocapnia with ketanserin, and hypercapnia with ketanserin were analyzed.

2.2.4. Data and statistical analyses

We monitored phrenic nerve activity as an index of central respiratory output. Samples were taken for analysis for each treatment, in each series, as indicated above. The moving averages of phrenic nerve discharge were quantified as: duration of burst (neural inspiration, T_I), inter-burst interval (the period between the bursts, the index of neural expiration, T_E), individual burst frequency (f_R: $60/(T_I + T_E)$, and burst peak amplitude (established as a neural index of tidal volume; nV_T; Eldridge 1971). Neural minute ventilation was calculated as the product of f_R and nV_T. The maximum response to CO_2 was evaluated by calculating the hypercapnic value as a proportion of normocapnic value. This was repeated with values obtained during drug exposure. Data

from both drug doses in the methysergide series (series I) were pooled as the responses were not significantly different from one another (Student's t-test). To determine if hypercapnia had an effect on neuroventilation, and whether or not a given pharmacological manipulation affected the magnitude of this response, two-way repeated-measures ANOVA were performed on absolute values for all variables except nV_T. Phrenic amplitude was recorded in arbitrary units. To establish the statistical significance of the effect of CO_2 on this parameter, a paired t-test was used on the normalized values. To evaluate the effect of pharmacological application on baseline firing, paired t-tests were used for all parameters. Data are expressed as mean ± SE. The criterion level for determination of statistical significance was set at $P < 0.05$.

2.3. Results

2.3.1. Eupnea

Baseline normocapnic values for parameters of phrenic nerve activity did not differ between experimental series (see Tables 2.1, 2.2 and 2.3 for individual series and group values). Under initial normocapnic conditions, phrenic bursts occurred at a mean rate of 29.9 ± 2.2 bursts/minute. The burst duration (T_I) was 0.46 ± 0.03 seconds and the period between bursts (T_E) was 1.78 ± 0.15 seconds. Bursts exhibited the typical incrementing pattern normally associated with eupneic integrated phrenic nerve discharge in this *in situ* preparation (Paton 1996; St-John and Paton 2000).

2.3.2. Series I: methysergide

Hypercapnic response

Four preparations were subjected to a hypercapnic challenge by increasing the level of CO_2 equilibrating the perfusate (Fig. 2.1). Neuroventilatory changes were limited to a significant augmentation in phrenic burst frequency (increase of 30 ± 3% above control) mediated entirely by a decrease in the duration of neural expiration (to 74 ± 1% of control). The burst length remained unaffected. There was a significant decrease in neural tidal volume as determined by peak phrenic amplitude (to 73 ± 8% of control). As

a result, the neural equivalent of minute ventilation (obtained by multiplying the integrated phrenic amplitude with burst frequency) was not altered in response to hypercapnia (93 ± 9% of control) (Fig. 2.2).

Drug effect on eupnea and the hypercapnic response

We administered methysergide to eight preparations, achieving final perfusate concentrations of 5 and 10 μM (n = 4 for each dose). Compared to control preparations, methysergide-treated preparations had greater burst frequency at baseline CO_2 (66 ± 18% greater than control) due to a lower T_E (to 58 ± 5 % of control) (Fig. 2.3). Importantly, frequencies greater than these values were routinely observed in drug-free hypercapnic conditions. Methysergide effects did not induce maximal burst frequencies. Peak amplitude was also slightly lower in methysergide-treated preparations. Responses to hypercapnia were statistically identical between the two methysergide doses; thus data for methysergide treatments were pooled. Hypercapnic responses were significantly different between methysergide-treated and control preparations (Fig. 2.1). When 5-HT_1 and 5-HT_2 receptors were inhibited, none of the phrenic burst parameters changed with an elevation of CO_2.

2.3.3. Series II: 8-OH-DPAT

Hypercapnic response

In a second set of experiments, exposure to hypercapnia resulted in a significant neuroventilatory response (n = 6). Neural minute ventilation was augmented 14 ± 5% above the control value (Fig. 2.2). Phrenic burst frequency increased by 12 ± 6%; however, no significant changes were observed in the period between bursts nor the burst length (Fig. 2.4). No consistent change in integrated peak phrenic amplitude was observed in conjunction with the increase in frequency with hypercapnia.

Drug effect on the hypercapnic response

Eupneic bursts continued following administration of 1.5 μM of 8-OH-DPAT. While the frequency of phrenic bursts was not altered, T_I was significant increased (to 15 ± 3 % above control) (Fig. 2.3). Peak amplitude was also significantly increased (to 14 ±

4% above control). Frequency response to hypercapnia in untreated preparations was absent in the presence of 8-OH-DPAT (Fig. 2.4). In addition, treatment with 8-OH-DPAT abolished the hypercapnic response of neural minute ventilation.

2.3.4. Series III: ketanserin

Hypercapnic response

Increasing the CO_2 equilibrating the perfusate resulted in a significant increase in phrenic burst frequency in this series (to 10 ± 4% above control) (Fig. 2.5). The elevated frequency was the result of a decrease in the period between bursts (to 89 ± 4% of control). The frequency response was accompanied by a significant increase in peak phrenic amplitude (to 8 ± 2% above control), resulting in a considerable increase in neural minute ventilation (to 20 ± 4% above control) (Fig. 2.2).

Drug effect on eupnea and the hypercapnic response

Ketanserin (5 μM) did not alter eupneic burst pattern in any of the eight preparations (Fig. 2.3). However, this dose was sufficient to abolish the neuroventilatory response previously evoked by exposure to hypercapnia (Fig. 2.5). When 5-HT_2 receptors were blocked, hypercapnia did not result in any significant changes in phrenic burst parameters.

2.4. Discussion

2.4.1. Chemosensitivity of the in situ preparation

Our primary objective was to determine if 5-HT neurons and 5-HT receptor activation are critical for central chemosensitivity. Thus, our first aim was to confirm that our experimental system, the arterially perfused *in situ* preparation, exhibited biologically relevant responses to hypercapnia. Such responses have been identified in previous studies (Day and Wilson 2005, 2007; St-John and Paton 2000). *In vivo*, animals respond to increases in arterial blood P_{CO_2} by altering ventilatory parameters such as breathing frequency and tidal volume. However, the relative changes in these parameters are

variable among species and across developmental stages within a single species. In adult rats, the ventilatory response to inhaling 7% CO_2 is an increase in minute ventilation caused by significant increases in both breathing frequency and tidal volume (Taylor et al. 2005). The response to high CO_2 in rats aged 30 days post-natal (P30) is similar to the adult response. A ~250% increase in minute ventilation is detected when compared to eucapnic values, due to both an increase in tidal volume (~200%) and breathing frequency (~130%) (Davis et al. 2006). Both central and peripheral chemoreceptors contribute to these responses, with approximately 60% of the magnitude of the hypercapnic ventilatory response due to central chemoreceptor activation (Smith et al. 2006). The perfused *in situ* brainstem preparation has deactivated peripheral chemoreceptors (Day and Wilson 2007) and would be expected to exhibit dampened chemosensitivity more commonly associated with peripherally denervated rodents (Gautier et al. 1993; Nattie et al. 1991). Additionally, the *in situ* preparation is decerebrate and vagotomized, both of which blunt the ventilatory response to hypercapnia *in vivo* (Martin-Body and Sinclair 1987; Nattie et al. 1991). Also, since the preparation is maintained at a mildly hypothermic temperature (31°C), neuroventilatory responses to CO_2 are expected to be diminished as is observed with hypothermic hypercapnia *in vivo* (Maskrey 1990). In general, with the impact of all these factors *in vivo*, the chemoresponses noted *in situ* are appropriate.

Both levels of hypercapnia (7 and 9% CO_2) elicited a significant hypercapnic response in the *in situ* preparation. Exposure to hypercapnia resulted in increases in neuroventilatory frequency in all experimental series. The 9% CO_2 treatment induced augmentation of neural minute ventilation, although this was not observed with 7% CO_2 treatment. The observed decrease in burst peak amplitude associated with hypercapnia (7% CO_2) present in the first series is perplexing. Although not a standard response, there are reports of similar paradoxical responses to hypercapnia in rats that may be strain-specific (Hodges et al. 2002). This tendency dictated that neural minute ventilation would not increase with 7% CO_2. In general, responses recorded in the current experiments are comparable to those observed in this preparation by other researchers (Day and Wilson

2005, 2007; St-John and Paton 2000). These results are consistent with hypercapnic responses reported for decerebrate, vagotomized, otherwise intact animals, but are blunted compared to fully intact animals (as discussed above).

The contributions of peripheral (carotid body-mediated) and central chemosensitivity have been determined in a variant of the *in situ* preparation (the dual perfused preparation, in which the peripheral and central chemoreceptive areas are independently perfused) (Day and Wilson 2007). The levels of CO_2 exposure normally used in this preparation (i.e. increasing CO_2 in the perfusate from 5% to 7%) fall on a relatively insensitive region of the CO_2 response curve. We took this to explain the relatively mild changes in neural ventilation observed with changes in CO_2 in our first series of experiments and as justification for the levels of CO_2 exposure chosen in subsequent series (5% to 9% CO_2).

Overall, our results illustrate that a significant and physiologically relevant neuroventilatory response occurs in our *in situ* preparations. This observation allows us to determine if 5-HT neurons and serotonergic receptor activation are critical for this chemosensitivity.

2.4.2. Methysergide abolished the hypercapnic ventilatory response

Methysergide maleate is a commonly used mixed 5-HT_1 and 5-HT_2 receptor antagonist. Previous use of this antagonist has produced varying effects on eupneic ventilation depending on the preparation type and experimental approach (Al-Zubaidy et al. 1996; Delpierre et al. 1994; Ptak et al. 2009; Toppin et al. 2007). One common influence of methysergide treatment is a depression of neural tidal volume, as illustrated in our results.

Our first series of experiments used this mixed receptor antagonist to determine if the 5-HT system plays a functional role in the hypercapnic neuroventilatory response *in situ*. Our results demonstrated that neuroventilatory responses to a CO_2 challenge were abolished by methysergide application. Methysergide is recognized as a 5-HT_1 and 5-HT_2 receptor antagonist (Hoyer et al. 1994). Our data suggest, therefore, that central chemosensitivity is critically dependent on 5-HT_1 and/or 5-HT_2 receptor activation. This

supports the hypothesis that 5-HT neurons, and the release of 5-HT, are necessary for central chemoreception.

2.4.3. 8-OH-DPAT facilitates neuroventilation

8-OH-DPAT is commonly used as a 5-HT_{1A} receptor agonist, which binds to somatodendritic 5-HT_{1A} receptors (autoreceptors). 5-HT_{1A} receptors are coupled to inhibitory G-proteins, causing an inhibition of adenylyl cyclase resulting in a decrease of cyclic adenosine monophosphate (cAMP) levels (Richter et al. 2003). This decreases neuronal excitability and causes hyperpolarization through activation of inwardly rectifying potassium channels and inhibition of N and P/Q-type calcium channels (Ladewig et al. 2004). Thus, ligand binding to somatodendritic 5-HT_{1A} receptors inhibits 5-HT release (McCall and Clement 1989; Sharp et al. 1989; Veasey et al. 1995).

8-OH-DPAT treatment in our *in situ* preparation augmented phrenic burst discharge by increasing T_I and peak amplitude. Given the role of 8-OH-DPAT in hyperpolarizing 5-HT neurons, our data suggest that 5-HT neurons limit T_I and peak amplitude during eupnea. Previous data from various animal preparations demonstrate conflicting results in ventilatory responses to 8-OH-DPAT, none of which are entirely consistent with the current results. 5-HT_{1A} agonism increases respiratory frequency in anesthetized adult rats and increases phrenic burst frequency in the reduced en bloc brainstem-spinal cord neonatal mouse preparation (Edwards et al. 1990; Hilaire et al. 1997). Whether the differences between reported responses and our current observations are due to dose or system-specific factors remains to be determined. Regardless, the impacts of 8-OH-DPAT on baseline activity in our preparation were not profound.

While the dominant role of 8-OH-DPAT is commonly assumed to be the silencing of 5-HT neurons, effects of this agonist on receptors other than 5-HT_{1A} autoreceptors should also be considered. Post-synaptic 5-HT_{1A} receptors are found in several respiratory-related nuclei including the hypoglossal motor nuclei, the retrotrapezoid nucleus, and the preBötC (Liu and Wong-Riley 2009; Okabe et al. 1997). The role of these post-synaptic 5-HT_{1A} receptors in respiration, however, is difficult to determine. Results using mice in which central 5-HT neurons are absent (and thus the action of 5-

HT_{1A} autoreceptors) suggest that activation of post-synaptic 5-HT_{1A} receptors with 8-OH-DPAT facilitates breathing (Corcoran et al. 2009b). As these receptors are coupled to inhibitory G-proteins, the mechanism for the facilitation on the respiratory rhythm is unknown. However, the respiratory-related effects of 8-OH-DPAT on post-synaptic receptors are more consistent with results reported in this study.

Additionally, 8-OH-DPAT has moderate affinity for 5-HT_7 receptors (Bard et al. 1993; Eriksson et al. 2008). While not traditionally found in respiratory nuclei, low levels of mRNA expression for 5-HT_7 receptors are found in the preBötC (Richter et al. 2003). Our knowledge of the role of 5-HT_7 receptors in breathing is very limited, although they do appear to be involved in restoring phrenic activity following a fentanyl-induced depression of respiratory discharge (Richter et al. 2003). The potential facilitation of respiration by activation of 5-HT_7 receptors could account for the increase in phrenic burst parameters that we observed. We speculate, therefore, that normocapnic responses to 8-OH-DPAT may reflect the actions of this agonist on systems other than 5-HT_{1A} autoreceptors.

2.4.3. The neuroventilatory response to hypercapnia is absent in the presence of 8-OH-DPAT

Phrenic burst frequency did not respond to hypercapnia during systemic application of 8-OH-DPAT, while it responded during control conditions. We interpret these data to suggest that central chemosensitivity is critically dependent on an 8-OH-DPAT-sensitive process. This agent attenuates 5-HT neuron activity through activation of 5-HT_{1A} autoreceptors (McCall and Clement 1989; Veasey et al. 1995). As such, these observations suggest that 5-HT neurons are critical for central chemosensitivity in our preparation. As discussed above, 8-OH-DPAT also impacts post-synaptic 5-HT_{1A} and 5-HT_7 receptors. Activation of both of these receptor subtypes appears to facilitate ventilation; thus a potential impact of 8-OH-DPAT on post-synaptic 5-HT_{1A} and 5-HT_7 receptors would not be expected to dampen hypercapnic sensitivity. We conclude, therefore, that these results most likely represent an inhibition of 5-HT neurons, critical

for chemosensitivity, through 5-HT_{1A} autoreceptors. This result further supports the hypothesis that 5-HT neurons are necessary for central chemoreception.

Recent evidence suggests heterogeneity in medullary raphé chemoreception. While Li et al. (2006) showed that dialysis of 8-OH-DPAT into particular sub-regions of the medullary raphé (within pallidus and obscurus) has no significant effect on the CO_2 response in awake and sleeping rats, Taylor et al. (2005) found that a similar manipulation targeting a more caudal area of the raphé (pallidus and magnus) attenuates the CO_2 response. The purpose of such experiments is to identify particular serotonergic nuclei involved in chemosensitivity, and potentially exclude others. Our experimental protocol involves more general activation of 5-HT_{1A} receptors and is not designed to draw distinction between 5-HT neuron populations involved in the hypercapnic ventilatory response. We show, however, that the 5-HT system is critical in this reflex.

2.4.4. A ketanserin-sensitive mechanism is critical for central chemosensitivity

We used ketanserin, a relatively specific 5-HT_2 antagonist, to identify the specific receptor subtype involved in the CO_2 response *in situ*. This receptor subclass, which is coupled to G-proteins and through IP_3- and DAG-inhibition of potassium channels induces neuronal excitability, and plays a critical role in respiratory-related rhythm generation and modulation *in vitro* (Pena and Ramirez 2002; Tryba et al. 2006). However, we must be cautious when interpreting the importance of 5-HT as a neuromodulator *in vitro*, as its role may be exaggerated in reduced preparations (Toppin et al. 2007). As such, ketanserin may have a different effect *in situ* than *in vitro*.

Application of ketanserin to the *in situ* preparation had little effect on eupneic phrenic nerve discharge, indicating both a lack of resting 5-HT_2-mediated serotonergic tone and no critical reliance of spontaneous eupneic patterns on 5-HT_2 receptor activation. 5-HT has been suggested to maintain stable breathing-related burst patterns *in vitro* (Pena and Ramirez 2002; Richter et al. 2003). Other studies, in more intact preparations, do not report a significant effect of 5-HT_2 receptor inhibition on breathing *in vivo* (Dias et al. 2007) or on eupneic neuroventilation *in situ* (Toppin et al. 2007). This discrepancy has been ascribed to a greater reliance on 5-HT_2 receptors in the generation

of stable repiratory-related discharge patterns *in vitro*, in the absence of other endogenous neuromodulatory factors (Toppin et al. 2007).

Ketanserin had a much more significant effect on the response to elevated CO_2. Addition of ketanserin to the aCSF abolished both the frequency and amplitude responses normally associated with an increase in CO_2 in the *in situ* preparation. This result illustrates that central chemosensitivity is critically dependent on a ketanserin-sensitive process. As this primary direct action of ketanserin is as an antagonist blocking the post-synaptic 5-HT_2 receptors, we conclude that 5-HT_2 receptor activation is critical for central chemosensitivity in our preparation. Again, this result lends further support to the hypothesis that 5-HT neurons are necessary for central chemoreception.

The application of ketanserin may have impacted not only 5-HT_2 receptors but also alpha-1 adrenergic receptors, as evidenced by its affinity for both receptors types (Hoyer et al. 1987). The disruption of central chemosensitivity in our preparation, therefore, may have resulted from a dependence of chemosensitivity on alpha-1 adrenergic receptors activation. However, as the primary outcome of the ketanserin treatment was similar to that of methysergide and 8-OH-DPAT (which do not target adrenergic receptors), the most parsimonious conclusion is that the resulting changes in the ventilatory hypercapnic response due to ketanserin were primarily due to inhibition of 5-HT_2 receptors rather than effects on the adrenergic system. This has been observed regarding ketanserin's hypotensive effect, where alpha-adrenergic blockade makes a minimal contribution compared to the serotonergic system (Naslund et al. 1988).

Ketanserin may also have some affinity for 5-HT_7 and histamine H1 receptors (Jasper et al. 1997; Shen et al. 1993; Wouters et al. 1985). Histamine plays a neuromodulatory role in breathing, with agonism of H1 receptors increasing phrenic burst frequency, and antagonists decreasing both frequency and burst amplitude (Dutschmann et al. 2003). We did not observe an effect of ketanserin on the eupneic phrenic burst frequency or amplitude in our preparations and therefore find it unlikely that ketanserin-induced antagonism of H1 or 5-HT_7 receptors greatly affected our results.

2.4.5. Controversy of the role of 5-HT neurons in hypercapnic sensitivity

Recently, the role of 5-HT neurons in the central response to CO_2 has been challenged (Guyenet 2008; Mulkey et al. 2004). In a recent review, we have disputed these arguments (Corcoran et al. 2009a). Prior to the present study, a weakness in the argument that 5-HT neurons function as chemosensors has been a lack of confirmation that 5-HT neuron-mediated processes are critical to ventilatory response to CO_2 in a relatively intact, non-anesthetized animal. Our present results provide further evidence that 5-HT neurons and 5-HT receptor activation are indeed important in the hypercapnic neuroventilatory response.

2.4.6. Conclusions

Our data demonstrated that, in an unanesthetized, decerebrate, and arterially perfused *in situ* preparation, administration of methysergide, 8-OH-DPAT, and ketanserin all prevented the neuroventilatory responses associated with hypercapnia. The feature unifying the pharmacological action of the three agents tested is an impact on serotonergic neurotransmission. Methysergide impacts 5-HT_1 and 5-HT_2 receptors; 8-OH-DPAT activates 5-HT_{1A} receptors, attenuating 5-HT neuron activity; and ketanserin blocks post-synaptic 5-HT_2 receptors. Taken together, our data suggest that activation of 5-HT neurons, releasing 5-HT as a neurotransmitter with impacts on post-syanptic 5-HT_2 receptors is critical for normal chemosensitivity. Our results are consistent with the growing pool of data suggesting that serotonin neurons, particularly those of the medullary raphé, play a large role in central chemoreception (Corcoran et al. 2009a; Dias et al. 2007; Hodges et al. 2008).

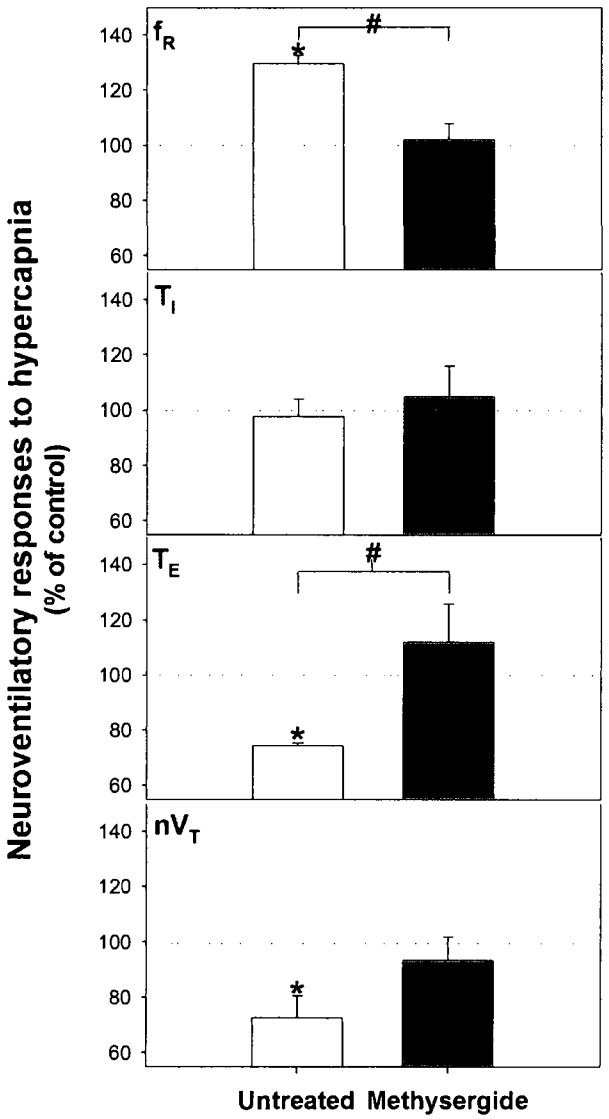

Figure 2.1 Phrenic nerve responses to hypercapnia without (untreated) and with exposure to methysergide (5-10 μM). Data are means ± SE of hypercapnic values expressed as a percentage of normocapnic control values. Significant differences from baseline are denoted by an asterisk (*) and a number sign (#) indicates a significant difference between untreated and treated groups ($n = 4$ for untreated and $n = 8$ for methysergide, two-way ANOVA, $P < 0.05$).

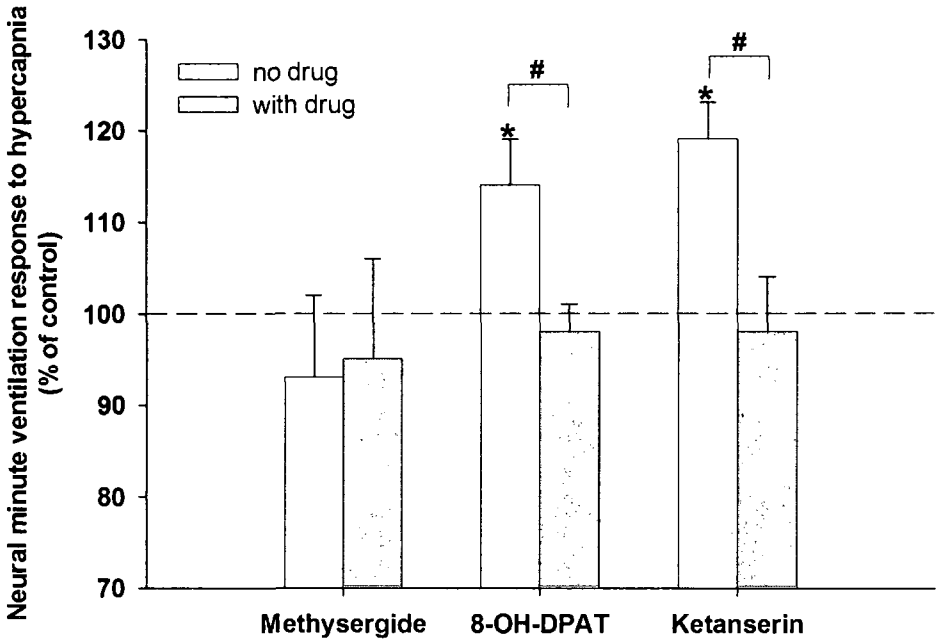

Figure 2.2 Effect of hypercapnia on neural correlates of minute ventilation (phrenic burst frequency × burst amplitude) before and during pharmacological manipulation of the serotonergic system. Data are means ± SE of hypercapnic values expressed as a percentage of normocapnic control values. Significant differences from baseline are denoted by an asterisk (*) and a number sign (#) indicates a significant difference between untreated and treated groups (two-way RM ANOVA, $P < 0.05$).

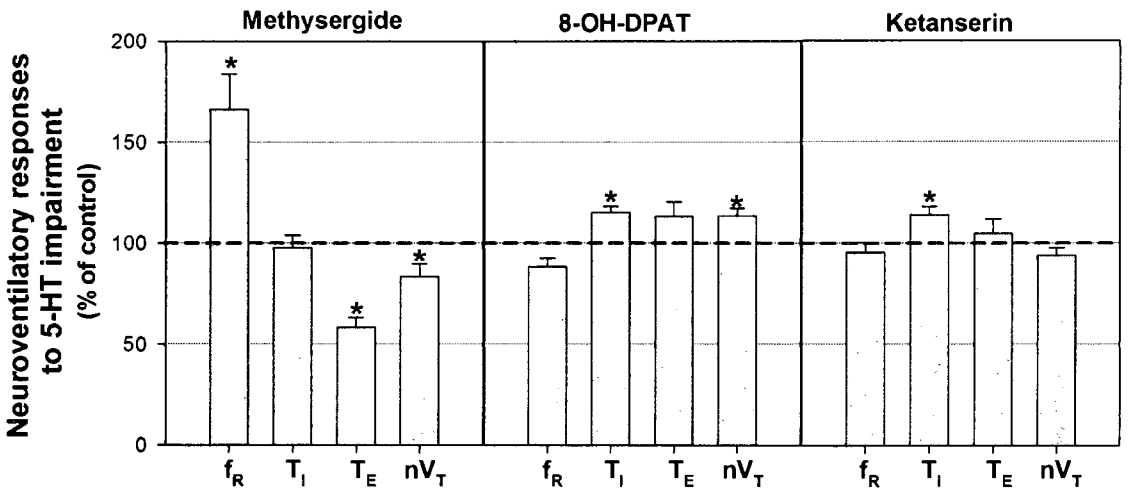

Figure 2.3 Effect of serotonergic antagonists and agonists on phrenic burst parameters in the *in situ* preparation. Data are means ± SE of burst parameter values during pharmacological administration expressed as a percentage of eupneic values. Significant differences from baseline are denoted by an asterisk (*) (paired t-test, $P < 0.05$).

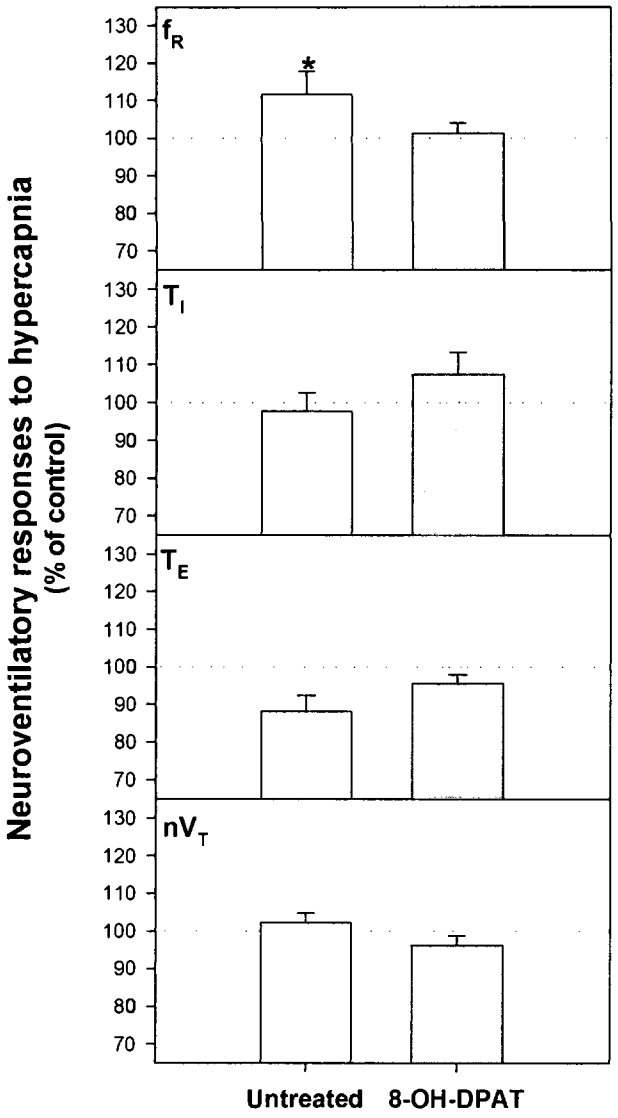

Figure 2.4 Phrenic nerve responses to hypercapnia without (untreated) and with exposure to 8-OH-DPAT (1.5 μM). Data are means ± SE of hypercapnic values expressed as a percentage of normocapnic control values. Significant differences from baseline are denoted by an asterisk (*) ($n = 6$; two-way RM ANOVA, $P < 0.05$).

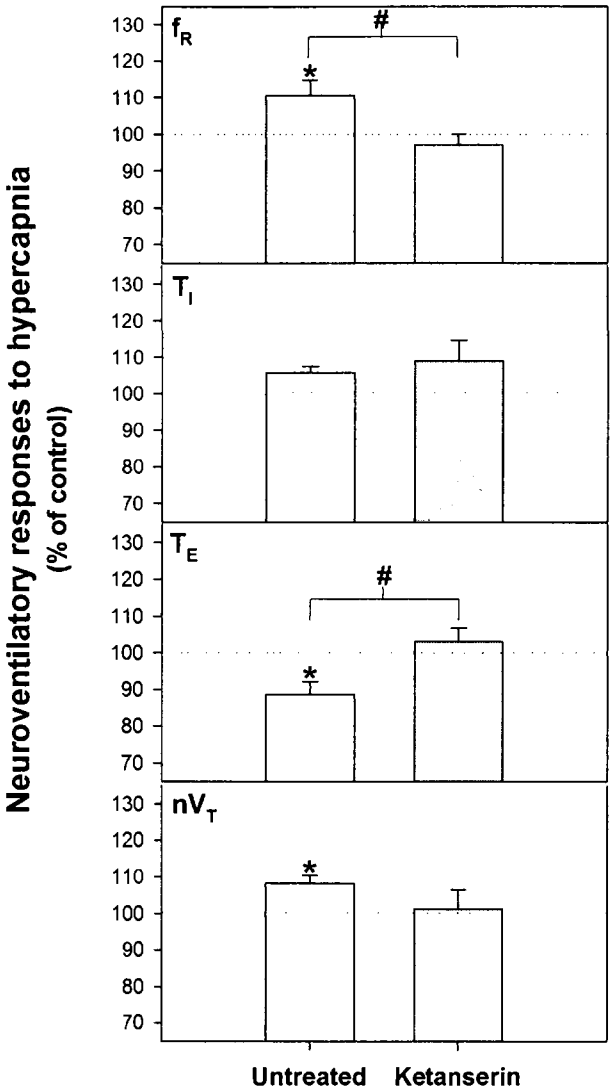

Figure 2.5 Phrenic nerve responses to hypercapnia without (untreated) and with exposure to ketanserin (5 μM). Data are means ± SE of hypercapnic values expressed as a percentage of normocapnic control values. Significant differences from baseline are denoted by an asterisk (*) and a number sign (#) indicates a significant difference between untreated and treated groups (n = 8; two-way RM ANOVA, $P < 0.05$).

Table 2.1 *Phrenic nerve frequency response to hypercapnia with and without 5-HT impairment*

			Phrenic burst frequency (min^{-1})			
			control		with treatment	
Treatment group		*n*	normocapnia	hypercapnia*	normocapnia	hypercapnia*
Methysergide**	control group	4	22.3 ± 1.1	28.9 ± 2.1	--	--
	drug group	8	21.4 ± 1.3	--	34.6 ± 2.5	34.6 ± 1.9
8-OH-DPAT		6	38.3 ± 4.2	41.5 ± 3.1	40.6 ± 1.9	41.4 ± 2.9
Ketanserin		8	27.3 ± 2.2	29.8 ± 1.8	30.3 ± 2.5	29.5 ± 2.7

Values are mean ± SE

* Methysergide preparations were exposed to 7% CO_2, and 8-OH-DPAT and ketanserin preparations to 9% CO_2

** Non-repeated design

Table 2.2 *Phrenic nerve burst duration (T_I) response to hypercapnia with and without 5-HT impairment*

			Phrenic burst duration (sec)			
			control		with treatment	
Treatment group		*n*	normocapnia	hypercapnia*	normocapnia	hypercapnia*
Methysergide**	control group	4	0.59 ± 0.07	0.59 ± 0.1	--	--
	drug group	8	0.47 ± 0.02	--	0.45 ± 0.02	0.48 ± 0.07
8-OH-DPAT		6	0.44 ± 0.03	0.43 ± 0.02	0.49 ± 0.02	0.53 ± 0.03
Ketanserin		8	0.40 ± 0.02	0.42 ± 0.02	0.47 ± 0.04	0.51 ± 0.05

Values are mean ± SE

* Methysergide preparations were exposed to 7% CO_2, and 8-OH-DPAT and ketanserin preparations to 9% CO_2

** Non-repeated design

Table 2.3 *Phrenic nerve inter-burst interval (T_E) response to hypercapnia with and without 5-HT impairment*

			Phrenic inter-burst interval (sec)			
			control		with treatment	
Treatment group		*n*	normocapnia	hypercapnia*	normocapnia	hypercapnia*
Methysergide**	control group	4	2.32 ± 0.08	1.72 ± 0.07	--	--
	drug group	8	2.50 ± 0.17	--	1.43 ± 0.12	1.51± 0.10
8-OH-DPAT		6	1.28 ± 0.27	1.07 ± 0.15	1.01 ± 0.09	0.97 ± 0.10
Ketanserin		8	1.89 ± 0.18	1.64 ± 0.12	1.62 ± 0.19	1.66 ± 0.19

Values are mean ± SE

* Methysergide preparations were exposed to 7% CO_2, and 8-OH-DPAT and ketanserin preparations to 9% CO_2

** Non-repeated design

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Chapter 3 Modulation of respiratory activity by hypocretin-1 (orexin A) *in situ* and *in vitro*[1]

Abstract

Release of hypocretins (orexins) by neurons in the lateral hypothalamus is an contributes importantly to arousal state, thermoregulation, and feeding behavior. Hypocretin neuromodulation has recently been proposed to play a role in breathing and central chemosensitivity. Using the *in situ* arterially perfused juvenile rat preparation, we determined the effect of hypocretin-1 (hcrt-1) and SB-408124 (an antagonist for hypocretin receptor subtype 1, hcrt-r1) on phrenic nerve activity, a neural correlate of breathing (neuroventilation), and on the neuroventilatory sensitivity to CO_2. Application of hcrt-1 through the perfusate had little effect on baseline firing. Blocking hcrt-r1, however, prevented the phrenic burst frequency response normally associated with hypercapnia. These data suggest that endogenous hypocretinergic modulation enhances neuroventilatory chemosensitivity. Further studies using the *in vitro* medullary slice preparation explored the effect of hcrt-1 on hypoglossal nerve activity, a correlate of ventilation *in vitro*. Application of exogenous hcrt-1 failed to significantly alter hypoglossal burst output in neonatal rat slices, indicating that this portion of the neuroventilatory circuit is insensitive to hcrt-1. Taken together, these data suggest that hcrt-1 is a modulator of central chemosensitivity.

3.1. Introduction

Hypothalamic neuropeptides hypocretin-1 (orexin A) and -2 (orexin B) are synthesized in cells of the lateral hypothalamus that project widely throughout the brain

[1] Corcoran AE, Richerson GB, and Harris MB. 2009. Modulation of respiratory activity by hypocretin-1 (orexin A) *in situ* and *in vitro*. Submitted to a Special Issue of Advances in Experimental Medicine and Biology.

(Peyron et al. 1998). Their extensive homeostatic roles include maintenance of arousal state, thermoregulation, and contributions to feeding behavior (Sakurai et al. 1998). Additionally, both anatomical and functional evidence suggests that endogenous hypocretins modulate breathing. Axons of orexin-containing neurons project to respiratory-related nuclei including the nucleus tractus solitarius (NTS), preBötzinger Complex (preBötC), and hypoglossal and phrenic nuclei, as well as central chemoreceptor areas such as the raphé nuclei (Fung et al. 2001; Nakamura et al. 2007; Peyron et al. 1998). The preBötC and the phrenic motor nuclei are immunoreactive for hypocretin receptor 1 (hcrt-r1) (Young et al. 2005), while hypocretin receptor 2 (hcrt-r2) is expressed in hypoglossal motoneurons (Volgin et al. 2002). Physiological evidence also indicates that hypocretins affect ventilation. Injection of hypocretin-1 into the lateral ventricle of urethane-anesthetized mice results in a small increase in breathing frequency (~11%) and a larger increase in tidal volume (~75%) (Zhang et al. 2005). Administration of hypocretin-1 into the preBötC *in vivo* increases diaphragmatic electromyogram activity but does not affect breathing frequency (Young et al. 2005). These results suggest that hypocretin stimulates respiration primarily through increases in tidal volume. Nakamura et al. (2007) looked at the respiratory phenotype of a prepro-orexin knockout mouse (which lacks both hypocretin-1 and -2). Frequency and tidal volume showed sleep-wake dependency in both wildtype and knockout mice; however, no significant differences in minute ventilation occur between the two phenotypes.

Three lines of evidence establish correlation between the hypocretinergic system and the hypercapnic chemoreflex. Hypocretin neurons themselves are chemosensitive *in vitro* (Sunanaga et al. 2009; Williams et al. 2007). Although ventilatory responses to hypercapnia do not differ between wildtype and prepro-orexin knockout mice during sleep states, these responses are blunted in knockouts during wakefulness (Nakamura et al. 2007). Antagonism of hcrt-r1 in the retrotrapezoid nucleus (RTN) blunts the response to hypercapnia, predominantly in wakefulness (Dias et al. 2009).

The aim of the current study was to identify the role of the hypocretinergic system in neuroventilation and the hypercapnic neuroventilatory response. Using the *in situ*

preparation, we identified the effects of hypocretin-1 administration and of hcrt-r1 antagonism on phrenic neuroventilation, and on changes in neuroventilation with hypercapnia (chemosensitivity). We also identified the effects of hypocretin-1 on the hypoglossal nerve output in a neonatal rat rhythmic medullary slice preparation. We tested the hypothesis that exognenous hypocretin-1 stimulates neuroventilation and enhances chemosensitivity, while antagonism of hcrt-r1 dampens neuroventilation and blunts chemosensitivity.

3.2. Methods

3.2.1. Arterially perfused in situ preparation

Experiments were performed on arterially perfused *in situ* preparations derived from juvenile (post-natal day 26-30; 60-100 g) male Sprague-Dawley rats. Details of this procedure have been published previously (Corcoran 2009; Paton 1996; Toppin et al. 2007). After one hour of baseline recording during perfusion with an artificial cerebrospinal fluid (aCSF; composed of, in mM: 1.0 $MgSO_4$, 0.25 KH_2PO_4, 4.0 KCl, 25 $NaHCO_3$, 115 NaCl, 2.0 $CaCl_2$, 10 dextrose, and 0.1785 Ficoll 70) equilibrated with 5% CO_2 balanced with 95% O_2 (normocapnia), CO_2 in the perfusate was increased to 10% for 5 minutes (hypercapnia). A comparison of the phrenic burst parameters recorded during hypercapnia relative to normocapnia illustrated the normal neuroventilatory response to hypercapnia. The perfusate was then re-equilibrated with 5% CO_2. Either hypocretin-1 (3 and 30 nM final concentration in aCSF; targets both hcrt-1r and hcrt-2r), SB-408124 (3 μM in aCSF, hcrt-r1 antagonist), or aCSF alone (sham treatment), was then used to perfuse the animal for 5 minutes prior to a second hypercapnic challenge completed in the presence of the pharmacological agent or sham. Differences in the response to hypercapnia were compared between the drug and control aCSF.

We attempted to determine the interaction between effects of hypercapnia and pharmacological modulation using a two-way repeated measures ANOVA; however, the data were not normally distributed, invalidating a critical assumption of this test, and precluding assessment in this manner. Statistical significance of the difference between

hypercapnia and baseline (either with or without pharmacological disruption) was resolved using Student's paired t-tests. Data presented are means ± SE. The criterion level for determination of statistical significance was set at $P < 0.05$.

3.2.2. In vitro medullary slice preparation

Experiments were performed on brainstem slices prepared from neonatal rats (post-natal day 0-4). Rats were decapitated, and the brainstem and spinal cord removed under a flow of chilled dissection solution composed of, in mM: 124 NaCl, 25 $NaHCO_3$, 3 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 0.5 NaH_2PO_4, 30 D-glucose, and equilibrated with 95% O_2 and 5% CO_2 (pH ~7.4). The brainstem was pinned to a wax block, and transverse slices of the medulla (550-650 μm thick) were prepared using a vibratome (see Smith et al. 1991 for details). Slices containing the preBötC, the hypoglossal motor nuclei, and the most rostral hypoglossal nerve rootlets were placed in a recording chamber (0.7 ml) and superfused with recording solution (dissection solution with elevated potassium) at a rate of 1.0 ml/minute via a syringe pump (Harvard Apparatus). The aCSF was warmed to 29°C using a temperature controller (Warner Instruments; TC-324B). Potassium concentrations were elevated in recording solutions (to 8 mM) to ensure rhythmic activity in the slice, as per standard procedures (Smith et al. 1991).

Inspiratory-related motor discharge of the hypoglossal rootlets was recorded continuously by glass suction electrodes. Signals were amplified x 10,000 and band-pass filtered (0.3 – 1 kHz) using a Grass LP511 AC amplifier and Dagan EX4-400 differential amplifier. A Hum Bug noise eliminator (Quest Scientific) was used to filter 60 Hz activity. Data were digitized at 100 kHz and analyzed off-line using Matlab software (MathWorks). Analysis of hypoglossal root discharge included an initial integration; full wave rectification and moving average over 50-msec periods.

After 30 minutes of baseline recording, the superfusate was switched to one containing hypocretin-1 (30 and 300 nM) and applied continuously to the slice for 10 minutes. Recordings during the last two minutes of hypocretin-1 application were used to determine the effect of the drug relative to baseline. Statistical significance was

determined using Student's paired t-tests. Data are presented as means ± SE. The criterion level for determination of statistical significance was set at $P < 0.05$.

3.3. Results

3.3.1. Effect of exogenous hypocretin-1 and hypocretin receptor antagonism on phrenic nerve activity

Neither dose of hypocretin-1 in the arterially perfused *in situ* preparation significantly altered baseline phrenic burst frequency nor the inter-burst interval (T_E) (Table 3.1; 3.2). The only effect observed was an increase in burst duration (T_I) following administration of 30 nM hypocretin-1 (increase from 0.48 ± 0.06 to 0.55 ± 0.06 seconds; Table 3.3). Exogenous hypocretin-1 did not affect burst amplitude.

Blockade of endogenous hypocretin on hcrt-r1 by SB-408124 (3 μM) significantly increased burst frequency (increase from 31.4 ± 2.0 to 33.9 ± 2.0 bursts/minute; Table 3.1). The increase in frequency was mediated by a decrease in T_E (decrease from 1.47 ± 0.09 to 1.33 ± 0.09 seconds), while no change in T_I was observed (Table 3.2; 3.3). SB-408124 did not alter baseline phrenic burst amplitude (not shown).

3.3.2. Effect of hypocretin-1 and hypocretin receptor antagonism on the neuroventilatory hypercapnic response

Increasing CO_2 in the perfusate significantly increased burst frequency in both control and treatment conditions (27 ± 4% increase from baseline in control; 16 ± 6% and 21 ± 4 % increases with 3 nM and 30 nM hypocretin-1, respectively; $n = 6$ for each dose; Fig. 3.1). This response was mediated by a decrease in T_E in each case (Fig. 3.1B). In all cases, hypercapnia did not alter burst amplitude or T_I (Fig. 3.1B).

A modest phrenic burst frequency response to hypercapnia was observed in the control group associated with SB-408124 treatment experiments (12 ± 4% increase from baseline, during control conditions; $n = 8$; Fig. 3.2). T_E was significantly decreased in response to hypercapnia in the absence of hcrt-r1 antagonists, while T_I was not altered.

Phrenic burst parameters or burst frequency did not change in response to hypercapnia once the hcrt-r1 antagonist was added (Fig. 3.2).

3.3.3. Effect of exogenous hypocretin-1 on hypoglossal nerve activity

Although there was substantial inter-slice variability, the hypoglossal burst activity of neonatal rat rhythmic medullary slices was unchanged by exogenous hcrt-1 treatment (Fig. 3.3). Hypoglossal burst frequency was not significantly different when exposed to 30 nM hypocretin-1 (from 5.7 ± 0.4 bursts/minute at baseline to 5.0 ± 0.4 bursts/minute with 30 nM hypocretin-1, $n = 10$). Similarly, exposure to 300 nM hypocretin-1 did not alter burst frequency (from 6.6 ± 0.9 bursts/minute at baseline to 5.5 ± 0.8 bursts/minute with 300 nM hypocretin-1, $n = 9$). Burst duration was not altered in response to either dose of hypocretin (30 or 300 nM: in seconds, 0.92 ± 0.05, 0.85 ± 0.08, baseline; 0.82 ± 0.06, 0.97 ± 0.07, treatment, respectively).

3.4. Discussion

3.4.1. Hypocretin and neuroventilation

Our data indicated that exogenous hypocretin had limited impact on neuroventilation *in situ* and no impact *in vitro*. Neither dose of hypocretin administered *in situ* had significant effect on baseline phrenic burst frequency. Similarly, hypocretin application in the *in vitro* rhythmic medullary slice had no effect on hypoglossal burst frequency. We interpret this lack of response to indicate that hypocretin had little to no impact on baseline neuroventilation, that hypocretin administered via systemic perfusate (*in situ*) was restricted from action within the central nervous system, or that sufficient endogenous hypocretin was present in the preparations such that addition of exogenous hypocretin had no impact.

The absence of a phrenic burst frequency response to exogenous hypocretin is consistent with some of the existing, albeit limited, literature. There is an anatomical basis for potential hypocretin-mediated alteration of respiratory rhythm as hypocretin-containing neurons project to the putative respiratory rhythm generator, the preBötC, and

hypocretin receptors are found on preBötC neurons (Young et al. 2005). Thus the anatomical potential for alteration of the respiratory rhythm is present. The reported effect of hypocretins on breathing frequency, however, is not consistent within the literature. Depending on the experimental design, hypocretins have little, if any, effect on breathing frequency. *In vivo* injection of exogenous hypocretin moderately increases breathing frequency (Zhang et al. 2005). However, direct application of exogenous hypocretin to the preBötC does not affect phrenic burst frequency (Young et al. 2005). In addition, mice with a genetically induced absence of endogenous hypocretin have similar breathing frequencies to wildtype mice (Nakamura et al. 2007).

The increase in phrenic burst frequency observed in response to SB-408124 is curious as it presumably resulted from blockade of endogenous hypocretin actions on hcrt-r1. The mechanism for such an effect is unknown, and it is not consistent with previous characterized actions of hypocretin on breathing frequency. This observation possibly reflects an unknown nonspecific action of SB-408124.

The primary modulation of ventilation by hypocretins is proposed to be through changes in tidal volume (Young et al. 2005; Zhang et al. 2005). Hypocretin receptors are found in phrenic motoneuron pools and hypocretinergic axons project to the phrenic nuclei (Fung et al. 2001; Young et al. 2005). We found no effect of exogenous hypocretin nor blockade of endogenous hypocretin on phrenic burst amplitude, a correlate of tidal volume (Eldridge 1971). The high dose of exogenous hypocretin (30 nM) used in the *in situ* preparation, however, increased burst duration. Burst duration correlates to the time devoted to inspiration, as phrenic nerve activity transmits to an inspiratory movement of the chest cavity through contraction of the diaphragm. An increase in inspiratory period, with exogenous hypocretin, would enhance inspiration and augment ventilation.

One factor potentially contributing to a lack of effects of hypocretinergic modulation (either by exogenous hypocretin, or by inhibition of hypocretin receptors) may be related to the circadian cycling of hypocretin. Hypocretin levels in the cerebrospinal fluid exhibit a circadian rhythm; levels of hypocretin-1 are high during wake and active periods, and low during sleep periods (Desarnaud et al. 2004). We do not

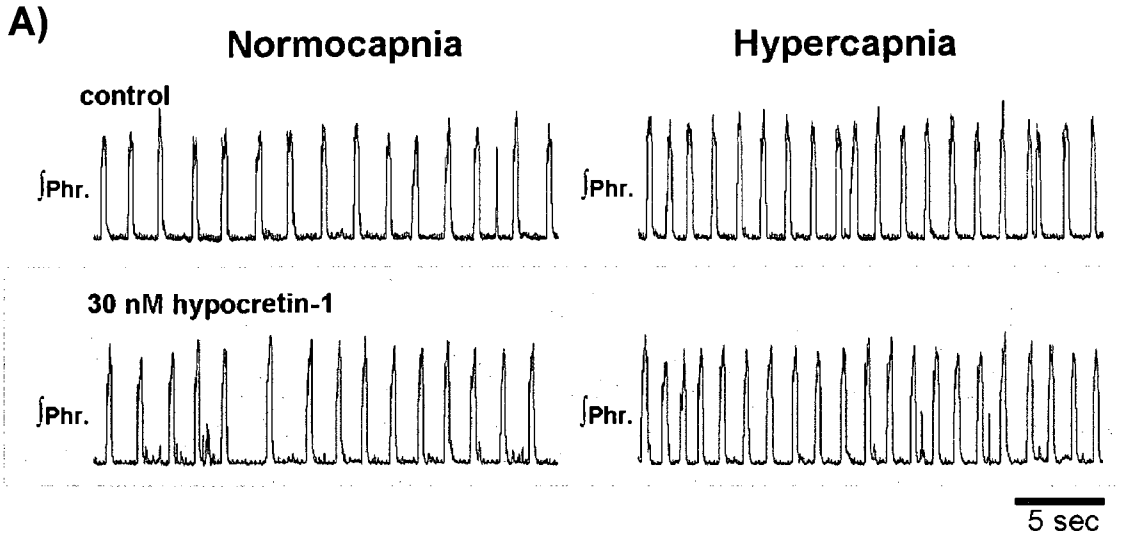
know the equivalent of arousal state in the *in situ* preparation, nor of the *in vitro* medullary slice preparation. It may be that the phase of the circadian cycle from which animals were taken could influence hypocretinergic responses of the *in situ* or *in vitro* preparations. Although we did no control for circadian cycle, individual responses did not appear to depend on the time of experiment (data not shown).

3.4.3. Effect of hypocretin receptor blockade on the hypercapnic neuroventilatory response

We hypothesized that since hypocretin appears to contribute to the hypercapnic ventilatory response (Dias et al. 2009; Nakamura et al. 2007), addition of hypocretin-1 could enhance the response seen *in situ*, and blockade of endogenous hypocretin using hcrt-r1 antagonists would blunt the response. We found that neuroventilatory sensitivity to CO_2 was not enhanced by exogenous hypocretin in the *in situ* preparation. Again, this suggests either that hypocretin was not involved with *in situ* chemoresponsiveness, or that endogenous hypocretin was sufficient. Since there was a neuroventilatory response to hypercapnia in the absence of a hcrt-r1 antagonist and there was no response in the presence of a hcrt-1 antagonist, we interpret the data to suggest that endogenous activation of the hypocretinergic system contributed to the ventilatory response to hypercapnia (though we were not able to statistically compare both responses).

Hypothalamic hypocretin-producing neurons are characterized as chemosensitive *in vitro* (Williams et al. 2007). Our data suggest, however, that hypocretin was not acting directly as a neurotransmitter transducing a chemosensory signal and activating respiratory control nuclei in our system. If CO_2-sensitive hypocretin neurons were responsible for hypercapnic neuroventilatory responses, through the release of hypocretin on respiratory-related nuclei, we would expect that exogenous hypocretin application in normocapnia would at least, in part, mimic neuroventilatory responses to hypercapnia. This was not the case. We postulate that the sensitivity and/or integration of central chemoreceptors is modulated by hypocretin, that endogenous hypocretin is sufficient to facilitate maximal sensitivity, and that it enhances normal sensitivity and/or integration of central chemoreceptors. Recently, hypocretin has been shown to contribute to

chemosensory integration within the RTN (Dias et al. 2009). Our data support a model where endogenous hypocretin facilitates central chemosensitivity in wakefulness, and disfacilitation associated with reduced hypocretin neuron activation during sleep reduces ventilatory chemosensitivity, and is normally associated with transitions from wakefulness to sleep (Estabrooke et al. 2001; Lee et al. 2005; Phillipson et al. 1977). Essential facilitation of chemosensitivity by hypocretin may also explain the relatively low sensitivity of putative chemosensitive neurons and circuits studied *in vitro*. We speculate that in the absence of critical endogenous hypocretin facilitation, *in vitro* systems exhibit reduced chemosensitivity when compared to greater chemosensitivity exhibited by more intact systems.



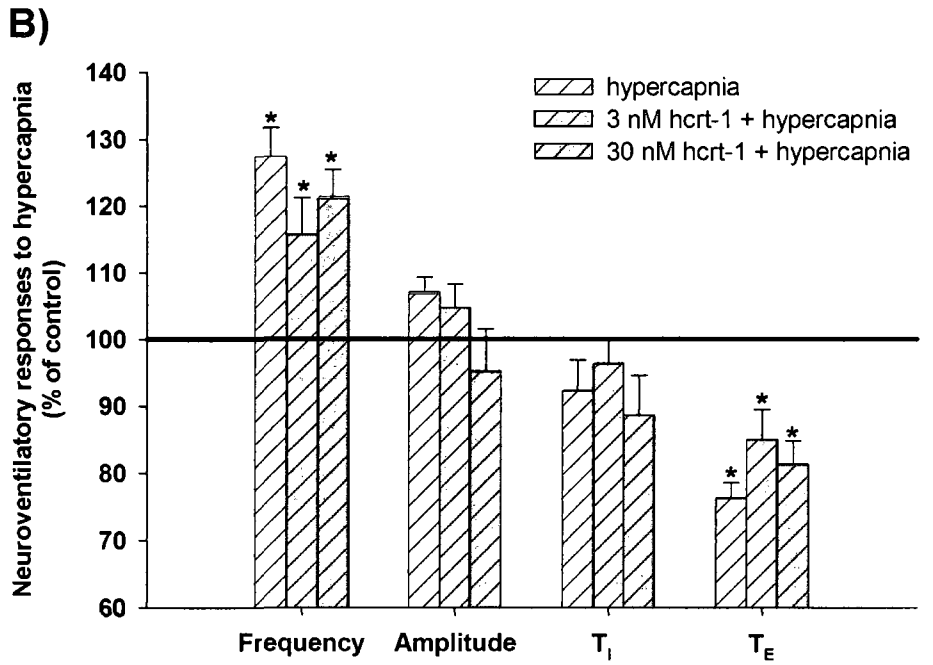


Figure 3.1 Effect of hypocretin-1 on the phrenic neuroventilatory response to hypercapnia in the perfused *in situ* rat preparation. A) Integrated traces at normocapnia and hypercapnia, before and during exposure to 30 nM hypocretin-1. B) Data are mean values ± SE expressed as a percentage of normocapnic control values before (white hatched bars), and after (grey hatched bars) administration of hypocretin-1 (3 nM and 30 nM; $n = 6$ for each dose). Significant differences from normocapnic baseline are denoted by * (paired t-test, $P < 0.05$). Abbreviations include: T_I, inspiratory time (burst duration); T_E, expiratory time (inter-burst interval).

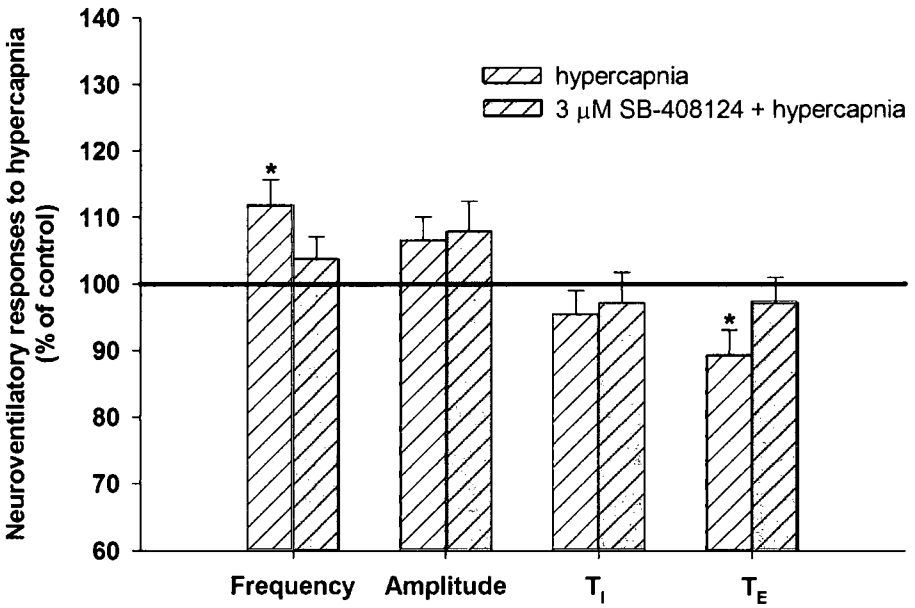


Figure 3.2 Effect of a hcrt-r1 antagonist (SB-408124) on the phrenic neuroventilatory response to hypercapnia in the perfused *in situ* rat preparation. Data are mean values ± SE expressed as a percentage of normocapnic control values before (white hatched bars), and after (grey hatched bars) administration of SB-408124 (3 μM; $n = 8$). Significant differences from normocapnic baseline are denoted by * (paired t-test, $P < 0.05$). Abbreviations include: T_I, inspiratory time (burst duration); T_E, expiratory time (inter-burst interval).

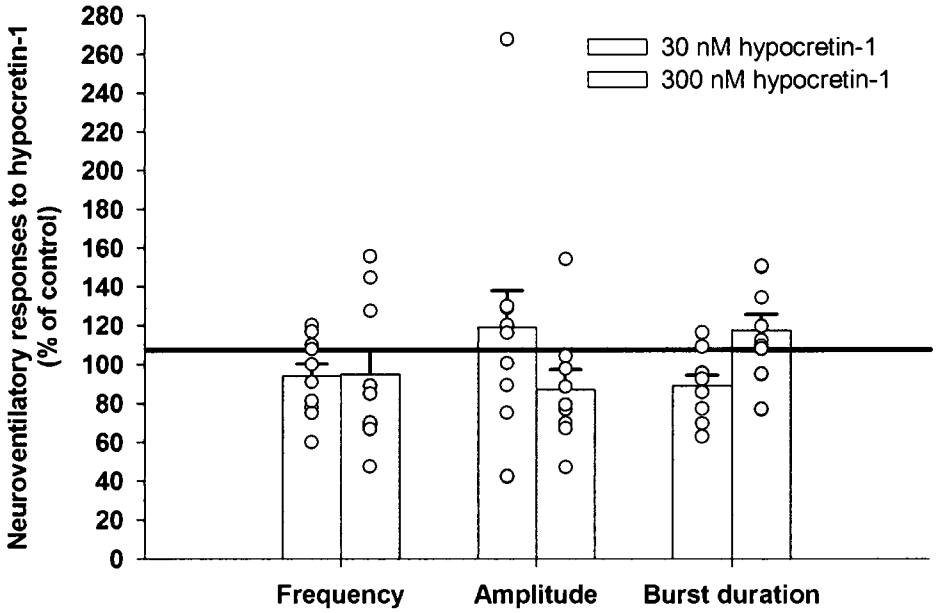


Figure 3.3 Effects of application of exogenous hypocretin-1 in neonatal rat medullary slice preparations. Shown are mean changes in burst frequency, amplitude, and duration in response to hypocretin-1(30 and 300 nM; n = 10 and 9, respectively). Data are mean values ± SE expressed as a percentage of normocapnic control values. Circles represent individual data points.

Table 3.1 *Phrenic nerve frequency response to hypocretinergic modulation during normocapnia*

			Phrenic burst frequency (min^{-1})	
Treatment group	Dose	*n*	Control	With treatment
hypocretin-1	3 nM	6	29.8 ± 0.7	30.8 ± 1.2
	30 nM	6	33.8 ± 5.3	31.5 ± 4.2
SB-408124	3 μM	8	31.4 ± 2.0	33.9 ± 2.0*

Values are mean ± SE

* indicates significant difference from control (paired t-test, $P < 0.05$)

Table 3.2 *Phrenic nerve burst duration (T_I) response to hypocretinergic modulation during normocapnia*

			Phrenic burst duration (sec)	
Treatment group	Dose	*n*	Control	With treatment
hypocretin-1	3 nM	6	0.43 ± 0.02	0.43 ± 0.03
	30 nM	6	0.48 ± 0.06	0.55 ± 0.06*
SB-408124	3 μM	8	0.49 ± 0.04	0.49 ± 0.03

Values are mean ± SE

* indicates significant difference from control (paired t-test, $P < 0.05$)

Table 3.3 *Phrenic nerve inter-burst interval (T_E) response to hypocretinergic modulation during normocapnia*

			Phrenic inter-burst interval (sec)	
Treatment group	Dose	*n*	Control	With treatment
hypocretin-1	3 nM	6	1.59 ± 0.04	1.53 ± 0.06
	30 nM	6	1.54 ± 0.30	1.52 ± 0.23
SB-408124	3 μM	8	1.47 ± 0.09	1.33 ± 0.09*

Values are mean ± SEM

* indicates significant difference from control (paired t-test, $P < 0.05$)

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Chapter 4 Functional link between the hypocretinergic and serotonergic systems in neuroventilation and central chemosensitivity[1]

Abstract

Serotonergic (5-HT) cells of the medullary raphé are putative central chemoreceptors, proposed to be one of multiple chemoreceptive sites in the brainstem interacting to produce the respiratory chemoreflex. Hypocretinergic neurons of the lateral hypothalamus, which are important contributors to arousal state, thermoregulation, and feeding behavior, are also reportedly involved in the hypercapnic ventilatory response. Recently, a functional interaction has been found between the hypocretinergic system and 5-HT neurons of the dorsal raphé. The validity and potential functional significance of hypocretinergic modulation of medullary raphé 5-HT neurons, however, is unknown. As such, the purpose of this study was to explore functional interactions between the hypocretinergic system and 5-HT system of the medullary raphé on both baseline respiratory-related output and central chemosensitivity. To explore such interactions, we took advantage of the *in vitro* medullary slice preparation derived from both wildtype (normal 5-HT function) and *Lmx1b*$^{f/f/p}$ neonatal knockout mice (lack all central 5-HT neurons) and examined effects of acidosis, hypocretin-1, hypocretin receptor antagonists (SB-334867, SB-408124, TCS OX2 29), and the effect of these antagonists on the response to acidosis. We confirmed the critical role of 5-HT neurons in central chemosensitivity as *Lmx1b*$^{f/f/p}$ preparations did not exhibit a response to acidosis, while wildtype increased hypoglossal burst frequency. We also found that hypocretins facilitated baseline neural ventilatory output in part through 5-HT neurons. While the impact of hypocretin on 5-HT neuronal sensitivity to acidosis is still in question, hypocretins did appear to mediate the burst duration response to acidosis via serotonergic mechanisms.

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[1] Corcoran AE, Richerson GB, and Harris MB. 2009. Functional link between the hypocretinergic and serotonergic systems in neuroventilation and central chemosensitivity. Prepared for submission to the Journal of Neurophysiology.

4.1. Introduction

Currently a growing body of literature suggests that serotonergic (5-HT) cells of the medullary raphé are an important chemoreceptive site in the brainstem (see Corcoran et al. 2009a and Richerson 2004 for review). Recent investigation into the importance of the 5-HT system in ventilatory chemosensitivity has exploited the use of a transgenic mouse in which a critical transcription factor (*Lmx1b)* is conditionally and selectively knocked out in all 5-HT cells in the central nervous system. This results in a near-complete (>99%) and specific absence of central serotonergic neurons as well as a coincident marked depletion of central 5-HT in the homozygote transgenic (Ding et al. 2003; Zhao et al. 2006). This $Lmx1b^{f/f/p}$ mouse is an invaluable tool as it enables us to focus more specifically on the role of 5-HT versus non-5-HT neurons in central chemosensitivity. As adults, *Lmx1b* knockouts breathe normally (albeit slightly slower) at rest, but they display a blunted ventilatory response to hypercapnia (about 50% less than wildtype mice) and abnormal thermoregulatory responses (Hodges et al. 2008). However, their response to elevated CO_2 as neonates has not been reported. While it is postulated that 5-HT neurons do not exhibit chemosensitivity in cell culture and slice until P12, chemosensitivity of these neurons may be altered in a rhythmic medullary slice preparation, derived from such neonatal mice, in response to a large change in pH. The present study is designed to illustrate the chemosensitivity mediated by neonatal 5-HT neurons, by quantifying the chemosensitivity of rhythmic slice preparations derived from mice with and without 5-HT neurons.

As more nuclei and areas are found to play a role in the respiratory chemoreflex, chemosensitivity is hypothesized as a distributed network function involving multiple chemoreceptive sites in the brainstem (Nattie and Li 2009). Progress is now being made to identify how these different chemoreceptive sites interact and modulate each other and/or the respiratory rhythm itself (Dias et al. 2008, 2009; Li et al. 2006; Mulkey et al. 2007).

A role for hypocretins in CO_2 sensitivity is a recently proposed idea (Dias et al. 2009; Nakamura et al. 2007). These neuropeptides, themselves recently discovered, are

synthesized by neurons in the lateral hypothalamus (Peyron et al. 1998; Sakurai et al. 1998). Hypocretin-1 (orexin A) and hypocretin-2 (orexin B) target two G-protein-coupled receptors; hypocretin receptor 1 (hcrt-r1) and hypocretin receptor 2 (hcrt-r2) (Sakurai et al. 1998). While hcrt-r1 is coupled to an excitatory G-protein, hcrt-r2 is coupled to either an excitatory or an inhibitory G-protein (Beuckmann and Yanagisawa 2002). The hypocretinergic system is anatomically linked with central respiratory nuclei as well as putative chemoreceptive sites (Fung et al. 2001; Hagan et al. 1999; Nakamura et al. 2007; Volgin et al. 2002; Young et al. 2005). The functional consequences of such interactions and the specific effects on ventilation remain unclear (Corcoran 2009; Nakamura et al. 2007; Young et al. 2005; Zhang et al. 2005). The emerging role of hypocretins in modulating the hypercapnic ventilatory response appears to be arousal-state dependent. Nakamura et al. (2007) shoed that while the response to inhaled hypercapnia is similar during sleep, CO_2 responses during wakefulness are blunted in hypocretin knockout mice compared to wildtype. This would suggest that hypocretins facilitate hypercapnic sensitivity specifically during the awake state. A link between hypocretins and chemoreception is also found in the retrotrapezoid nucleus (RTN), illustrated by a state-dependent effect of hypocretins on the hypercapnic ventilatory response; antagonism of hcrt-r1 in the RTN blunts the response to hypercapnia predominantly in wakefulness (Dias et al. 2009).

Similar potential interactions between the hypocretinergic system and the medullary raphé have not yet been explored. To date, most of the physiological evidence linking the hypocretinergic system with the 5-HT system has focused on the dorsal raphé and the regulation of sleep and wakefulness (hypocretin-1 excites 5-HT dorsal raphé neurons both in brainstem slices and *in vivo*; Brown et al. 2001; Takahashi et al. 2005). However, since hypocretin receptors 1 and 2 are also present in the medullary raphé, and projections of hypocretin neurons terminate in that area, it is possible that this system interacts with the medullary 5-HT system to promote a response to hypercapnia (Marcus et al. 2001; Nambu et al. 1999; Zheng et al. 2005).

The aims of the present studies were two fold. The first aim was to test the hypothesis that 5-HT neurons contribute to the neuroventilatory response to low pH in an *in vitro* rhythmic medullary slice preparation. To do this, we compared responses to acidosis in slice preparations derived from wildtype (containing 5-HT neurons) and *Lmx1b*$^{f/f/p}$ (lacking central 5-HT neurons) mouse strains. The second aim was to establish that hypocretinergic modulation of neuroventilation and chemosensitivity is determined by an impact of hypocretin on 5-HT neurons. We tested the hypothesis that hypocretins contribute to neuroventilation and promote the neuroventilatory response to acidosis through 5-HT neurons. To do this, we identified whether hypoglossal nerve activity changes generated by hypocretin-1 and hypocretin receptor antagonists depended on the presence of 5-HT neurons. We also identified the effects of hypocretin receptor antagonism on hypoglossal nerve responses to acidosis and whether or not this was dependent on the presence of 5-HT neurons.

4.2. Methods

All procedures and protocols were approved by the Institutional Animal Care and Use Committees of Yale University, where this research was conducted.

4.2.1. Animal model

The generation of *Lmx1b*$^{f/f/p}$ mice has been previously described by Zhao et al. (2006). Briefly, the breeding strategy involves mating females homozygous for floxed *Lmx1b* (*Lmx1b*$^{f/f}$) with males homozygous for floxed *Lmx1b* and hemizygous for *ePet1-Cre* (*Lmx1b*$^{flox/flox,ePet\text{-}Cre/+}$, also referred to as *Lmx1b*$^{f/f/p}$) to create offspring that lack both *Lmx1b* alleles specifically in Pet1-Cre expressing cells (5-HT neurons). This strategy results in a 1:1 ratio of wildtype (from here on referred to as WT; containing central 5-HT neurons) and *Lmx1b*$^{f/f/p}$ pups (lacking >99% of central 5-HT neurons). *Lmx1b* is a transcription factor that is expressed during the embryonic period and is required for 5-HT neuron differentiation (Ding et al. 2003). In *Lmx1b*$^{f/f/p}$ mice, the *Lmx1b* allele is flanked by two lox P sites (which contain specific binding sites for Cre) and is excised in cells that express the Cre protein (a site-specific DNA recombinase of loxP sequences).

Since the Cre protein is expressed only in cells that also express Pet-1 (a transcription factor only in cells destined to be 5-HT neurons), the *Lmx1b* alleles are knocked out specifically in Pet-1 neurons. Genotyping was determined by tail tissue samples obtained from each mouse and using procedures described by Zhao et al. (2006).

4.2.2. Experimental preparation

Experiments were performed on brainstem slices prepared from WT and $Lmx1b^{f/f/p}$ littermates (post-natal day 1-5) with the investigator blind to the genotype. Mice were decapitated and the brainstem and spinal cord removed under a flow of chilled artificial cerebrospinal fluid (aCSF) composed of, in mM: 124 NaCl, 25 $NaHCO_3$, 3 KCl, 1.5 $CaCl_2$, 1.0 $MgSO_4$, 0.5 NaH_2PO_4, 30 D-glucose, and equilibrated with 95% O_2 and 5% CO_2 (pH ~7.4). The brainstem was pinned to a wax block, and transverse slices of the medulla (550-600 μm thick) were prepared using a vibratome (see Smith et al. 1991 for details). Slices containing the preBötzinger Complex (preBötC), the hypoglossal motor nuclei, and the most rostral hypoglossal nerve rootlets were placed in a recording chamber (0.7 ml) and superfused with aCSF at a rate of 0.7 ml/minute via a syringe pump (Harvard Apparatus). The aCSF was warmed to 29°C using a temperature controller (Warner Instruments; TC-324B). Potassium concentrations were elevated to 9 mM to ensure rhythmic activity in the slice, as per standard procedures (Smith et al. 1991).

Inspiratory-related motor discharge of the hypoglossal rootlets was recorded continuously by glass suction electrodes. Signals were amplified x10,000 and filtered (0.3 – 1 kHz) using a Grass LP511 AC amplifier and Dagan differential amplifier (EX4-400). A Hum Bug noise eliminator (Quest Scientific) was used to reduce 60 Hz noise. Data were digitized at 100 Hz and analyzed off-line using Matlab software (MathWorks). Analysis of hypoglossal root discharge included an initial integration; full wave rectification and moving average over 50-msec periods.

4.2.3. Pharmacological agents

All pharmacological agents were obtained from Tocris, stored in stock solution at -20°C, and thawed the day of an experiment. The following agents were added to the

aCSF to produce the following final concentrations: hypocretin-1 (Orexin A) (300 nM), TCS OX2 29 (3 μM), SB-334867 (3 μM) and SB-408124 (3 μM). The hypocretin receptor antagonists (TCS OX2 29, SB-334867 and SB-408124) were all dissolved in DMSO prior to being added to the aCSF, with the final concentration <0.005% DMSO. After 30 minutes of baseline recording, the superfusate was switched to one containing either hypocretin-1, SB-408124, or TCS OX2 29 and SB-334867 (these last two agents were co-applied) and applied continuously to the slice for 10 minutes. To administer an acidotic challenge, the superfusate (pH 7.4) was switched to one of pH 6.9 for 10 minutes. Low pH was achieved prior to experiments by adding HCl to the gas-equilibrated aCSF (95% O_2 and 5% CO_2) and continuously monitoring changes in pH with a pH electrode until the value was stable at 6.9.

4.2.4. Data analysis

Integrated hypoglossal nerve discharge was analyzed to determine burst frequency, peak burst amplitude, burst duration, duration of the period between bursts (inter-burst interval), and the coefficient of variation of the inter-burst interval (CV IBI; calculated as the standard deviation / mean; an indication of timing variability; Hodges et al. 2009). Values were averaged from the last two minutes of baseline immediately prior to treatment and the last two minutes of treatment (either pH change or pharmacological agent). All data are presented as mean ± SE or as expressed as a proportion of the baseline value. Statistical significance was evaluated using one-way or two-way ANOVA with repeated measures, followed by Dunn's or Bonnferroni's *post hoc* analyses for multiple comparisons, where appropriate, to compare effects of pH, genotype, or drug, and their interactions. Additionally, differences between genotype group means of baseline burst parameters were compared using a Student's t-test. The criterion level for determination of statistical significance was set at $P < 0.05$ for all data analyses.

4.3. Results

4.3.1. Genotype-induced differences in hypoglossal burst parameters

Under baseline conditions, respiratory-related burst patterns differed between WT and *Lmx1b*$^{f/f/p}$ preparations (Table 4.1, Fig. 4.1A). These data were combined from baseline periods of all experimental series (n = 21 WT and 19 *Lmx1b*$^{f/f/p}$). Hypoglossal bursts occurred at 6.8 ± 0.5 bursts/minute in *Lmx1b*$^{f/f/p}$ slices compared to 9.2 ± 0.9 in WT. Although this apparent trend matched previous characterizations of reduced burst frequencies in *Lmx1b*$^{f/f/p}$ preparations (Corcoran et al. 2009b), no statistically significant difference between the two genotypes in the present dataset was found (P = 0.055). Burst duration was similar between the two genotypes. Respiratory-related output was severely disrupted in *Lmx1b*$^{f/f/p}$ as indexed by the coefficient of variation of the interburst interval. This parameter was significantly elevated in *Lmx1b*$^{f/f/p}$ compared to WT (P < 0.001), indicating significant variability in the hypoglossal burst pattern of *Lmx1b*$^{f/f/p}$ preparations, also apparent in representative tracings (Fig. 4.1A). This finding was consistent with our previous datasets (Corcoran et al. 2009b).

4.3.2. pH differentially affects hypoglossal burst parameters in WT and Lmx1b$^{f/f/p}$ *preparations*

Following baseline observations at pH 7.4, preparations were exposed for 10 minutes to acidotic aCSF with pH 6.9 (Fig. 4.1). This acidosis increased hypoglossal burst frequency in WT slices (57 ± 15% change from baseline; n = 12; Fig. 4.1B). Frequency increased gradually once the superfusate containing pH 6.9 reached the slice chamber, and after 5 minutes the frequency was significantly elevated (Fig. 4.1C). The latency in this response is likely caused by the time required for the recording chamber to equilibrate to the pH change, and for the acidic aCSF to diffuse into the tissue. Acidosis also increased burst duration (18 ± 5% change from baseline), while burst amplitude was unchanged. Acidosis significantly altered burst pattern regularity; the coefficient of variation of the interburst interval was reduced (78 ± 8% of baseline).

Responses to acidosis in *Lmx1b*$^{f/f/p}$ preparations differed from those of WT preparations. Most notably, acidosis did not increase burst frequency in *Lmx1b*$^{f/f/p}$ preparations. Like in WT preparations, acidosis did not alter burst amplitude, but did increase burst duration in *Lmx1b*$^{f/f/p}$ preparations (25 ± 5% increase from baseline; n = 12). Acidosis greatly increased burst regularity in *Lmx1b*$^{f/f/p}$ preparations; the coefficient of variation of the inter-burst interval was reduced (50 ± 6% of baseline), a significantly greater decrease than occurred in WT preparations (Fig. 4.1B).

In both WT and *Lmx1b*$^{f/f/p}$ preparations, effects of pH change were reversible and burst parameters returned to baseline values within 10-15 minutes of reperfusion with normal aCSF (pH 7.4). This switch, however, was accompanied by a significant transient decrease in burst frequency observed only in *Lmx1b*$^{f/f/p}$ preparations (5 minutes post-acidosis exposure); however, there was no statistical difference between WT and *Lmx1b*$^{f/f/p}$ preparations (Fig. 4.1C).

4.3.3. Exogenous hypocretin affects hypoglossal burst parameters

The most consistent observation in response to exogenous hypocretin-1 (300 nM), in both WT and *Lmx1b*$^{f/f/p}$ genotypes, was an increase in tonic activity in the hypoglossal nerve (Fig. 4.2A). Exogenous hypocretin-1 had little effect on baseline burst parameters (Fig. 4.2). The only significant effect in WT preparations (n = 7) was a decrease in hypoglossal burst frequency (to 67 ± 11% of baseline). This effect was less pronounced in *Lmx1b*$^{f/f/p}$ preparations (n = 8; Fig. 4.2B). Exogenous hypocretin-1 did increase regularity of the hypoglossal burst pattern in *Lmx1b*$^{f/f/p}$ preparations, as indicated by a significant reduction in the CV IBI (to 70 ± 9% of baseline). Effects of hypocretin-1 were reversible with washout and burst parameters returned to baseline values within 10-15 minutes.

4.3.4. Blockade of endogenous hypocretin affects hypoglossal burst parameters

We examined the influence of endogenous hypocretin receptor activation by determining the effect of hypocretin receptor antagonism on hypoglossal nerve output using two experimental series with different antagonists (Fig. 4.3). In the first series we

co-applied SB 334867 (a hcrt-r1 antagonist) and TCS OX2 29 (a hcrt-r2 antagonist; Fig. 4.3A) because a mixed hcrt-r1/-r2 antagonist was not available at the time of these experiments. The second series involved application of a single alternative hcrt-r1 antagonist (SB-408124; Fig. 4.3B). In both WT and *Lmx1b*$^{f/f/p}$ preparations, co-application of hypocretin antagonists significantly decreased burst frequency. This effect was more pronounced in WT (reduction to 45 ± 4% of baseline) compared to a more modest reduction (to 80 ± 9% of baseline) in *Lmx1b*$^{f/f/p}$preparations (n = 12 for each genotype). In the second series, SB-408124 had very similar effects on hypoglossal burst frequency (reduction to 38 ± 7% of baseline in WT, and to 61 ± 6% of baseline in *Lmx1b*$^{f/f/p}$ preparations, respectively; Fig. 4.3B; n = 6 for each genotype). Again, the reduction in frequency with hypocretin antagonism was more severe in WT preparations. Neither antagonist treatment altered burst amplitude. Both antagonist applications increased burst duration in WT preparations (by 49 ± 13% above baseline, and 68 ± 22% above baseline, for SB-334867 + TCS OX2 29 and SB-408124 treatments, respectively). Blockade of hcrt-r1 alone using SB-408124 lengthened bursts in *Lmx1b*$^{f/f/p}$ preparations (by 41 ± 14% from baseline; Fig. 4.3B)

4.3.5. Effect of hypocretin receptor antagonism on the response to acidosis

We examined the influence of hypocretin receptor antagonists on pH sensitivity by exposing slices to solutions with pH 7.4 and 6.9 both without and with hypocretin receptor antagonism. Figure 4.4 illustrates changes in hypoglossal burst frequency in response to acidosis observed under control conditions (normal aCSF) and blockade of hcrt-r (when aCSF contained either SB-334867 + TCS OX2 29 or SB-408124) alone, in both WT and *Lmx1b*$^{f/f/p}$ preparations. Despite blockade of hypocretin receptors, WT preparations exhibited a frequency response to acidosis (increase from 3.4 ± 0.6 to 8.9 ± 2.0 bursts/minute in the presence of both antagonists; increase from 3.0 ± 0.8 to 8.2 ± 1.8 bursts/minute in the presence of SB-408124 alone; n = 6 for each drug treatment). Switching to pH 6.9 restored the frequency, dampened by antagonists, to a level equivalent to baseline control values (control pH 7.4). In *Lmx1b*$^{f/f/p}$ preparations, no significant frequency response to a change in pH from 7.4 to 6.9 in the presence of

hypocretin antagonists was observed. This paralleled the responses in the absence of antagonists (n = 7 for co-application of SB-334867 + TCS OX2 29; n = 6 for SB-408124). Hypocretin antagonism blocked the change in burst duration otherwise observed with acidosis in WT preparations (Table 4.2); however, this may have been due to an increase in burst duration due to the antagonists themselves (Fig. 4.3). In contrast, burst duration increased with a switch to pH 6.9 (see Table 4.2 for changes relative to values at pH 7.4) in both the presence and absence of hypocretin receptor antagonists in *Lmx1b*$^{f/f/p}$ preparations. Similar to responses to acidosis alone, burst amplitude did not change in response to acidosis in either genotype while hypocretin receptors were blocked.

4.4. Discussion

4.4.1. Lmx1b$^{f/f/p}$ *preparations do not respond to acidosis*

One of the major findings of the current study was that, unlike the response normally exhibited in WT preparations, *Lmx1b*$^{f/f/p}$ preparations failed to increase hypoglossal burst frequency in response to acidosis. The *Lmx1b*$^{f/f/p}$ mouse, a conditional knockout, is a useful model for studying the role of 5-HT neurons in ventilatory control, including the contribution of 5-HT neurons to central chemosensitivity. Recent work has shown that in adults, breathing is relatively stable in *Lmx1b*$^{f/f/p}$; however, the hypercapnic ventilatory response is decreased by ~50% compared to WT (Hodges et al. 2008). In contrast, neonates display severely disordered breathing, which can be restored with addition of agonists for 5-HT$_{2A}$ and NK-1 receptors (Hodges et al. 2009). Chemosensitivity has not yet been studied in *Lmx1b*$^{f/f/p}$ mice at young ages. Our data demonstrated that, while WT rhythmic medullary slices produced a very robust response to a severe change in pH (from 7.4 to 6.9), the frequency of hypoglossal bursts was not significantly altered in *Lmx1b*$^{f/f/p}$ preparations. As the difference between WT and *Lmx1b*$^{f/f/p}$ mice is an almost complete absence of 5-HT neurons, these data provide further evidence that 5-HT neurons are critical in the ventilatory response to hypercapnia. Our results demonstrate that 5-HT neurons are critical in generating a frequency response

to acidosis. While finding 5-HT neurons that are chemosensitive prior to P12 is rare (Wang and Richerson 1999; Wu et al. 2008), the evidence of the importance of 5-HT-related chemosensitivity in our neonatal preparations is clear. Generally 5-HT neurons produce a large response to a change in pH from 7.4 to 7.2 (Wang et al. 2002). 5-HT neurons might be chemosensitive at younger ages but may require a larger stimulus.

An interesting observation was made during the return to control pH (7.4) after exposure to acidosis. *Lmx1b*$^{f/f/p}$ slices showed a significant decrease in hypoglossal burst frequency during recovery from a low pH. This overshoot compared to initial baseline suggesting that reversing an acidotic challenge activates chemosensitive receptors that inhibit respiratory-related output. A likely candidate may be GABAergic mechanisms previously reported in the medullary raphé. Richerson (1995) suggests two distinct populations of raphé neurons that differentially respond to CO_2 in a slice preparation and which could alter respiration. Subsequent research has identified two subtypes of chemosensitive neurons located in the ventromedial medullary raphé, 'acidosis-stimulated' neurons (positively identified as 5-HT neurons) and 'acidosis-inhibited' neurons (Wang et al. 1998, 2002). In addition, acidosis-inhibited neurons are stimulated by hypocapnia and alkalosis and are thus considered 'alkalosis-stimulated' neurons. The identity of these alkalosis-stimulated neurons of the medullary raphé has been characterized as GABA-synthesizing neurons (Corcoran et al. 2008). It is possible that the lack of 5-HT neurons in the *Lmx1b*$^{f/f/p}$ mice leads to an absence of what would normally be an acidosis-stimulated and alkalosis-inhibited neuromodulation of ventilation, revealing a ventilatory depression mediated by activation of alkalosis-stimulated GABAergic neurons during the transition from pH 6.9 to 7.4. Definitive conclusions cannot be drawn, however, regarding the potential masking of GABAergic inhibition as no significant differences in frequency were found between *Lmx1b*$^{f/f/p}$ and WT preparations.

4.4.2. Hypocretin-1 effects on baseline bursting

Exogenous hypocretin-1 increased tonic hypoglossal nerve activity in both WT and *Lmx1b*$^{f/f/p}$ preparations. The significance of this observation is unclear. Hypocretin

receptors have been localized to a number of motor neuron pools (Fung et al. 2001; Young et al. 2005), and this result may be due to general motorneuron activation. Tonic hypoglossal nerve activity was not different between WT and *Lmx1b*$^{f/f/p}$ preparations, indicating that 5-HT neurons were not involved in this process.

Beyond the general tonic activation, exogenous hypocretin-1 had little effect on baseline hypoglossal burst parameters. One curious result was the decrease in hypoglossal burst frequency in WT preparations. The physiological relevance of this effect is unclear. The observation that the rhythmic slice was relatively insensitive to exogenous hypocretin suggests that the rhythmogenic circuits present in the *in vitro* slice are not normally modulated by hypocretin or that sufficient endogenous hypocretin was present in these preparations such that addition of exogenous hypocretin had no impact. This conclusion is also supported by previous observations that exogenous hypocretin does not consistently alter phrenic burst discharge in the juvenile arterially perfused rat preparation, nor the hypoglossal nerve output in a rhythmic rat medullary slice (Corcoran 2009).

Exogenous hypocretin-1 enhanced the regularity of the hypoglossal burst pattern in *Lmx1b*$^{f/f/p}$ preparations. Since this tendency was only observed in the *Lmx1b*$^{f/f/p}$ slices, preparations which lack central 5-HT neurons and were more irregular at baseline compared to WT, it suggests that 5-HT is necessary for stability of the respiratory-related rhythm (also supported by evidence from Hodges et al. 2009; Pena and Ramirez 2002; Richter et al. 2003). In addition, this instability in *Lmx1b*$^{f/f/p}$ slice preparations can be rescued by exogenous hypocretin, although the mechanism is unknown. Thus, 5-HT neuron contribution to the stability of the respiratory rhythm can be by-passed by a hypocretinergic mechanism. Other contributors that appear to restore breathing stability caused by a lack of 5-HT include substance P and elevated potassium (Hodges et al. 2009).

Anatomical and functional evidence indicate that endogenous hypocretins modulate breathing. Axons of hypocretin-containing neurons project from the hypothalamus to respiratory-related nuclei including the nucleus tractus solitarius (NTS),

preBötC, the locus coeruleus, and hypoglossal and phrenic nuclei (Fung et al. 2001; Hagan et al. 1999; Nakamura et al. 2007; Young et al. 2005). Also, areas corresponding to the preBötC and the phrenic motonuclei are immunoreactive for hcrt-r1 receptors (Young et al. 2005) and hypoglossal motor neurons express hcrt-r2 (Volgin et al. 2002). Information from patients with various respiratory disorders also hint at a correlation between breathing and hypocretins. Patients with Guillain-Barre syndrome, a syndrome often associated with respiratory paralysis, have low levels of hypocretin in their cerebrospinal fluid (Ripley et al. 2001). Patients with sleep apnea have low blood levels of hypocretin (Sakurai et al. 2005).

In addition, physiological experiments indicate that hypocretins enhance ventilation. Injection of hypocretin-1 into the lateral ventricle of a urethane-anesthetized mouse results in a small increase in breathing frequency (~11%) and a larger increase in tidal volume (~75%) (Zhang et al. 2005). Administration of hypocretin-1 into the preBötC increases diaphragmatic electromyogram activity but does not affect breathing frequency (Young et al. 2005). This is suggestive that hypocretin stimulates respiration primarily through increases in tidal volume (Young et al. 2005). Nonetheless, the importance of the role of the hypocretinergic system in breathing remains unclear, as the respiratory phenotype (both breathing frequency and tidal volume) of a knockout mouse that lacks both hypocretin-1 and -2 is not significantly different than WT (Nakamura et al. 2007). Our findings indicated that exogenous application of hypocretin-1 (which targets both types of hypocretin receptors) reduced hypoglossal burst frequency in WT medullary slice preparations, while this treatment had no influence in *Lmx1b*$^{f/f/p}$ slices. This result is in contrast with more intact animal studies listed above, where hypocretin either had no effect on breathing frequency or increased it. This discrepancy may be due to influences not present in the reduced slice preparation.

4.4.3. Hypocretin antagonists have more severe effects on hypoglossal activity in WT

Addition of hypocretin antagonists to the aCSF resulted in changes in the hypoglossal burst output of both WT and *Lmx1b*$^{f/f/p}$ preparations. As hypocretin

antagonists should have an influence only if hypocretin receptor activation is present, we conclude that endogenous hypocretin was still present in the slices.

Hypocretin neuropeptides are synthesized exclusively in the lateral hypothalamus (Sakurai et al. 1998), an area that is absent in the *in vitro* rhythmic medullary slice preparation. However, these hypothalamic neurons project widely throughout the brainstem (Peyron et al. 1998), and it is feasible that a quantity of endogenous hypocretin is stored at synaptic terminals. This would explain how addition of hypocretin receptor antagonists could have significant effects in a preparation that lacks hypocretin neuronal cell bodies. The sources of endogenous hypocretin, blocked by antagonists, are unknown. We speculate that the elevated potassium concentrations used in the perfusate may induce spontaneous synaptic release of hypocretin despite the isolation of hypocretin nerve terminals, present in the slice, from their hypothalamic cell bodies. An alternative explanation, however, is that the antagonists used bind to other types of receptors. Co-application of antagonists for both hypocretin receptors resulted in a similar reduction in hypoglossal burst frequency as did blockade of hcrt-r1 alone (Fig. 4.3). Thus, facilitation of the respiratory-related rhythm by endogenous hypocretin is likely primarily through activation of hcrt-r1. An alternative explanation, however, is based on the differential expression of hypocretin receptors during development. While hcrt-r1 gene expression gradually increases from P0 to P25, expression of hcrt-r2 is not detected in the paraventricular nucleus (a site of high hcrt-r2 expression in adult rats) at P0 or P1, is only weakly expressed at P5, and then gradually increases until P20 (Yamamoto et al. 2000). While this could explain our current results, one must be cautious of such interpretation as these experiments were performed in rats (while ours were conducted in mice), and these species may differs in developmental expression of hypocretin receptors.

The effects of hypocretin antagonism were more profound in WT than *Lmx1b*$^{f/f/p}$ preparations. Again as the differences between these two genotypes are associated with a lack of central 5-HT neurons in *Lmx1b*$^{f/f/p}$ mice, these data suggest that facilitation of the respiratory rhythm by hypocretin was mediated, in part, through activation of 5-HT neurons. An anatomical basis for such interactions exists. Low density expression of

both hcrt-r1 and hcrt-r2 is found in the raphé magnus, while in the raphé obscurus hcrt-r1 is modestly expressed and no expression of hcrt-r2 is present (Marcus et al. 2001). Also, hypocretin axon terminals are found in the raphé magnus and pallidus (Nambu et al. 1999). A much denser innervation of hypocretin-1 fibers is observed in the raphé pallidus (Zheng et al. 2005). While neurophysiological recordings have not been made from the medullary raphé, hypocretin-1 depolarizes dorsal raphé 5-HT neurons (Brown et al. 2001; Takahashi et al. 2005). If the influences of hypocretin antagonism are dependent on hypocretin receptor activation on raphé 5-HT neurons, then the lack of such neurons in *Lmx1b*$^{f/f/p}$ slices would explain the limited impact of hypocretin in this genotype.

4.4.4. Hypocretin affects the pH response

We also sought to determine whether hypocretins modulate the ventilatory response to acidosis, and if so, whether this is through excitation of 5-HT neurons. To do this we compared hypoglossal burst responses to low pH in rhythmic medullary slices derived from wildtype and *Lmx1b*$^{f/f/p}$ mice before and during hypocretin receptor antagonism. While baseline (pH 7.4) frequency was markedly lower in preparations where hypocretin receptors were blocked, burst frequency increased in a similar fashion in response to pH 6.9 as did the control slices (which lacked the hypocretin receptor antagonists). This suggests that hypocretin did not enhance or promote the response to acidosis. Such a result is in contrast to our data from arterially perfused *in situ* rats, where systemic administration of SB-408124 inhibited the ventilatory hypercapnic response (Corcoran 2009), and other reports that hypocretins modulate chemosensitivity (Dias et al. 2009; Nakamura et al. 2007). Yet to be determined impacts of species (rat vs mouse) and age (juvenile vs neonate) may contribute to such responses. The most likely basis for the difference, however, is that the elements responsible for the observation that hypocretin receptor antagonist-mediated attenuation of chemosensitivity occur outside the rhythmic slice.

Since acidosis did not induce a change in hypoglossal burst frequency in *Lmx1b*$^{f/f/p}$ preparations, the lack of response in the presence of hypocretin receptor

antagonists is not unexpected and does not give us any information as to the importance of hypocretin in such a response.

The only evidence alluding to a hypocretinergic facilitation of the hypercapnic response due to an interaction with the 5-HT system is based on changes in hypoglossal burst duration. In WT preparations, bursts were significantly longer during exposure to pH 6.9 than during exposure to pH 7.4. Blockade of hypocretin receptors eliminated this response. In *Lmx1b*$^{f/f/p}$ preparations, a similar lengthening in hypoglossal bursts was observed at pH 6.9 compared to pH 7.4; however, hypocretin receptor antagonism did not inhibit this response as it did in WT. How and whether this increase in duration is mediated by 5-HT neurons remains unknown.

4.4.5. Implications for arousal

We cannot assess the arousal state in an *in vitro* slice preparation and, therefore, cannot directly determine the link between arousal, hypocretin, and 5-HT. This may be of consequence as much evidence indicates that involvement of the hypocretinergic system in the hypercapnic ventilatory response is arousal-state dependent; hypocretin plays a more significant role during wakefulness (Dias et al. 2009; Nakamura et al. 2007). This complements observations that hypocretin neurons fire spontaneously during wake periods and less, or not at all, during sleep (Estabrooke et al. 2001; Lee et al. 2005). Given the anatomical and physiological evidence linking the hypocretinergic and serotonergic systems, and the involvement of both of these systems in arousal, they possibly interact to promote a response to hypercapnia. Lack of a physiological correlate of arousal may explain why hypocretin antagonism does not alter the hypoglossal burst frequency response to acidosis in the medullary slice preparation.

In summary, we have shown that 5-HT neurons are required for the frequency response to severe acidosis in the *in vitro* rhythmic medullary slice preparations, furthering evidence that they are critically involved in central chemoreception. Exogenous application of hypocretin-1, as well as hypocretin receptor antagonism, differentially alters respiratory-related output in WT and *Lmx1b*$^{f/f/p}$ preparations, suggesting that 5-HT neurons contribute to the role of hypocretin in fictive ventilation.

While we do not have evidence that hypocretin contributes to the frequency response to acidosis, this neuropeptide appears to facilitate the acidosis-induced increase in hypoglossal burst duration via modulation of 5-HT neurons.

Figure 4.1. Acidosis differentially affects hypoglossal burst parameters in rhythmic slices from neonatal wildtype (WT) and *Lmx1b*$^{f/p}$ mice. A) Raw and integrated traces from both genotypes at normal pH (7.4) and during acidosis (pH 6.9). *Lmx1b*$^{f/p}$ bursting is more irregular and generally slower compared to WT during control conditions. B) Mean changes in burst frequency, amplitude, duration, and coefficient of variation of the interburst interval (CV IBI) in response to acidosis. Data are mean values ± SE expressed as a percentage of control values. Acidosis increases burst frequency in WT preparations only. C) Time course of mean burst frequency ± SE in both genotypes during control conditions (pH 7.4), acidosis (pH 6.9), and washout (ie. return to normal aCSF). * indicates a statistically significant difference from baseline levels, and † indicates a significantly different response in the two genotypes (n = 12 for each genotype; B) two-way RM ANOVA, C) one-way RM ANOVA; $P < 0.05$).

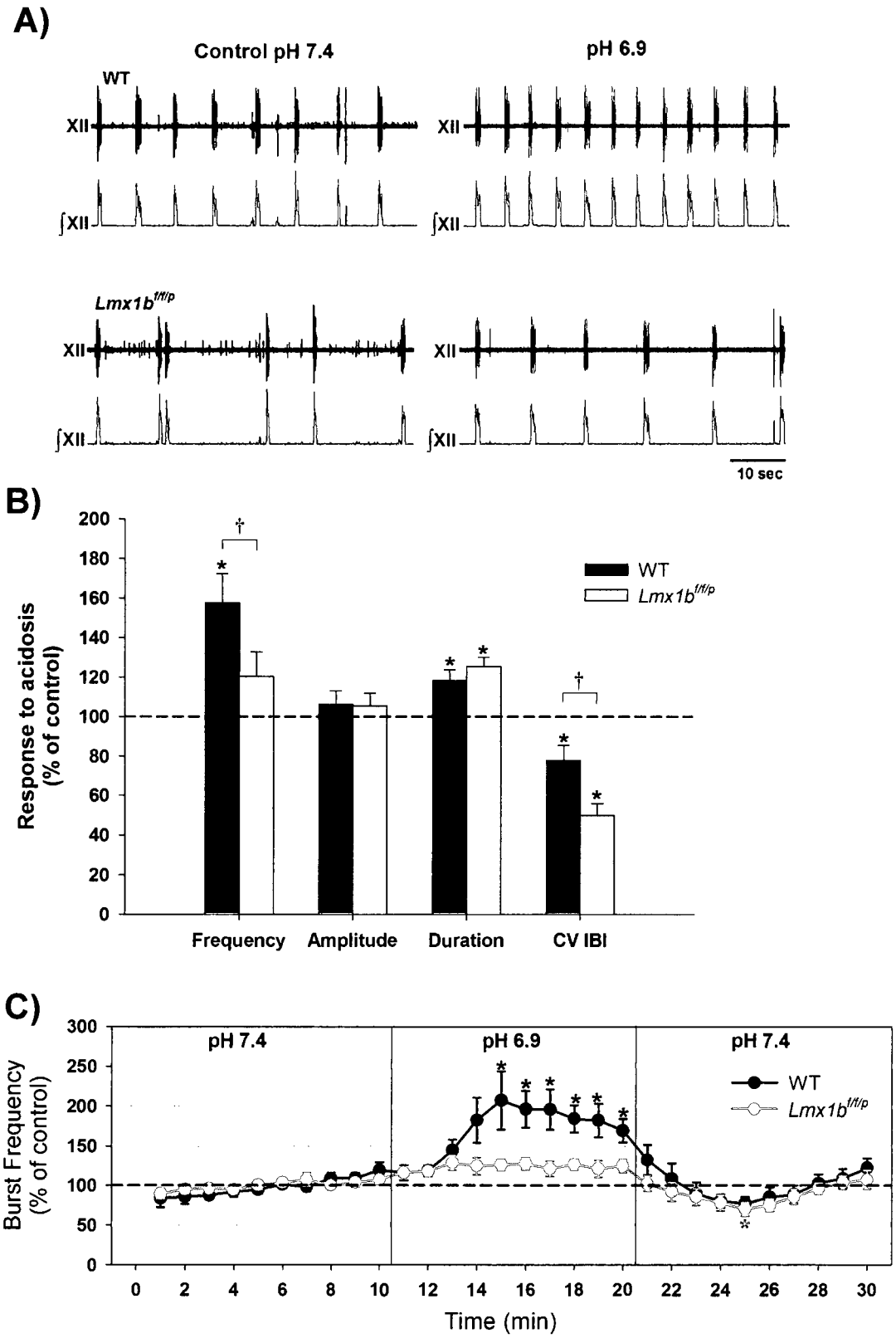
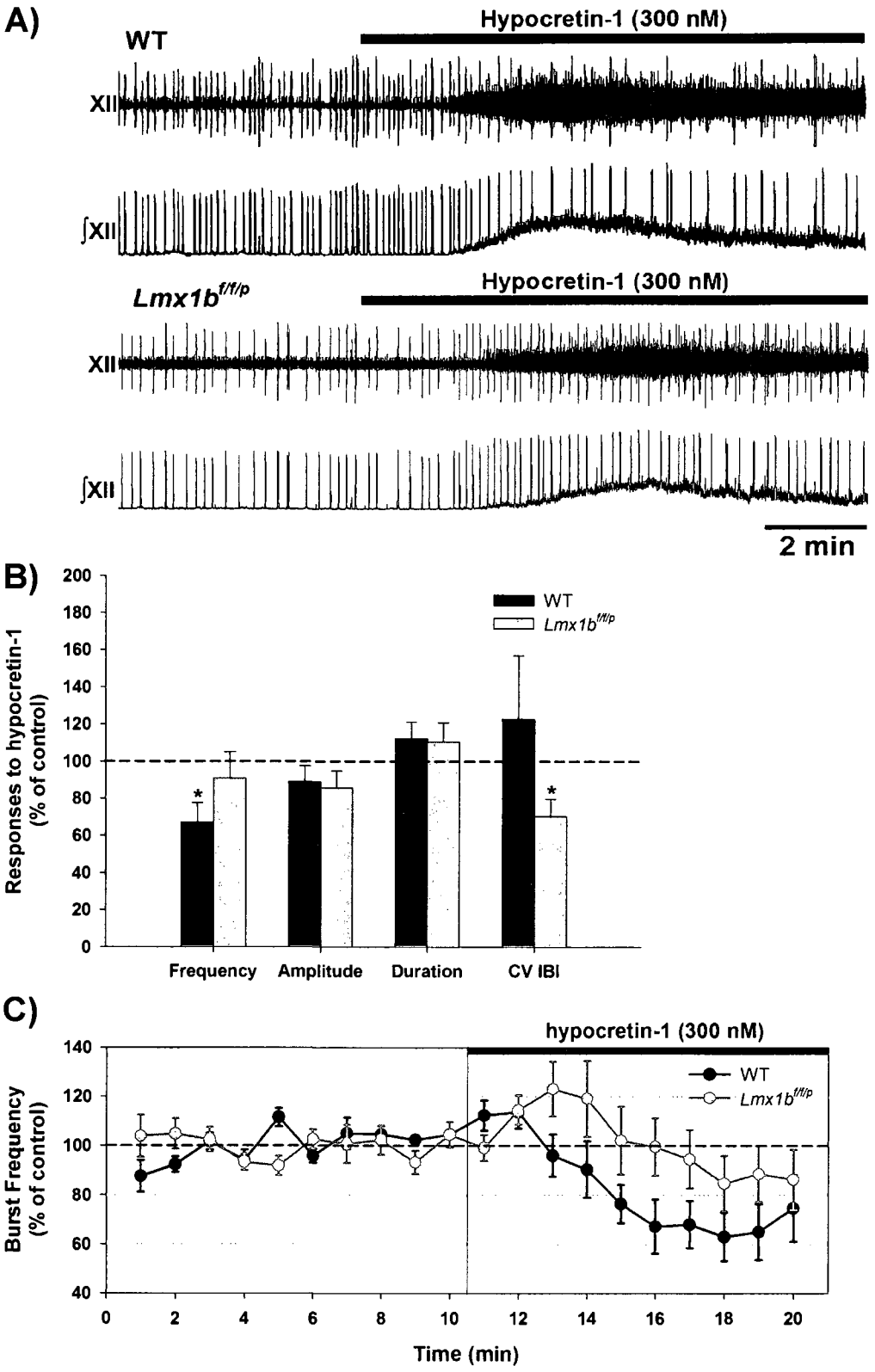

A)
Control pH 7.4
pH 6.9
WT
XII
∫XII
Lmx1b f/f/p
10 sec
B)
Response to acidosis (% of control)
WT
Lmx1b f/f/p
Frequency
Amplitude
Duration
CV IBI
C)
pH 7.4
pH 6.9
pH 7.4
Burst Frequency (% of control)
Time (min)

Figure 4.2. Effects of application of exogenous hypocretin-1 (300 nM) in wildtype (WT) and *Lmx1b*$^{f/p}$ rhythmic slices. A) Raw and integrated traces from both genotypes perfused with normal aCSF and during exposure to hypocretin. An increase in tonic activity was commonly observed in the presence of exogenous hypocretin. B) Mean changes in burst frequency, amplitude, duration, and coefficient of variation of the interburst interval (CV IBI) in response to hypocretin. Data are mean values ± SE expressed as a percentage of control values. Exogenous hypocretin reduced instability in *Lmx1b*$^{f/p}$ preparations. C) Time course of burst frequency in both genotypes during control conditions and during application of hypocretin. * indicates a statistically significant difference from baseline levels (ie. no hypocretin; $n = 7$ for WT and $n = 8$ for *Lmx1b*$^{f/p}$; two-way RM ANOVA, $P < 0.05$).


A)
WT
Hypocretin-1 (300 nM)
XII
∫XII
Lmx1b f/f/p
Hypocretin-1 (300 nM)
XII
∫XII
2 min
B)
Responses to hypocretin-1 (% of control)
WT
Lmx1b f/f/p
Frequency
Amplitude
Duration
CV IBI
C)
hypocretin-1 (300 nM)
Burst Frequency (% of control)
WT
Lmx1b f/f/p
Time (min)

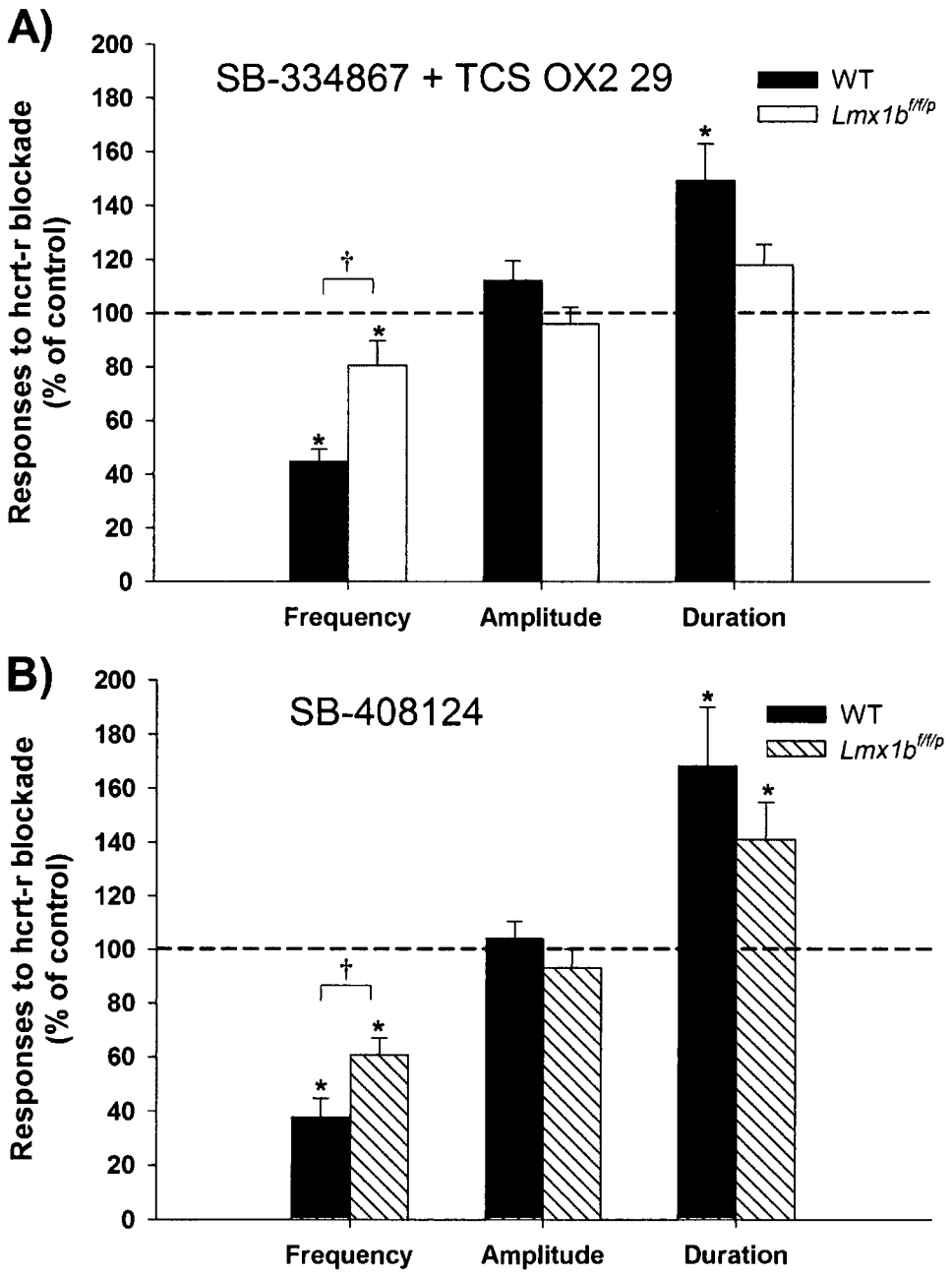


Figure 4.3. Hypocretin receptor antagonism effects on hypoglossal burst frequency, amplitude, and duration. A) Responses to co-application of hcrt-r1 and hcrt-r2 antagonists (SB-334867 and TCS OX2 29, respectively; 3 μM each; n = 12 for each genotype). B) Responses to a different hcrt-r1 antagonist, SB-408124 (3 μM; n = 6 for each genotype). Data are mean values ± SE expressed as a percentage of control. * indicates a statistically significant difference from control, and † indicates a significantly different response between the two genotypes (2-way-RM-ANOVA, $P < 0.05$).

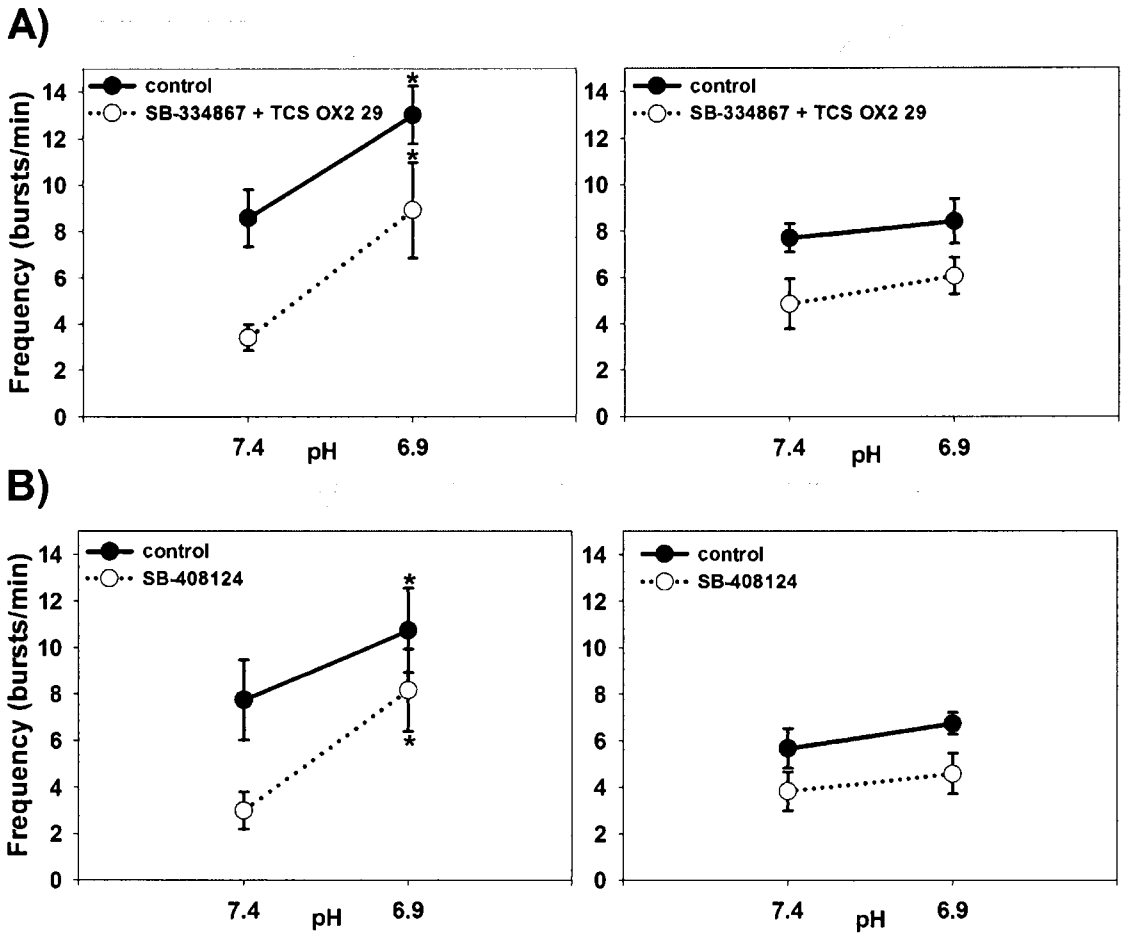


Figure 4.4. Burst frequency response to pH 6.9 and the impact of hcrt-r1 and hcrt-r2 antagonists on this response in WT and *Lmx1b*$^{f/f/p}$ preparations. A) Co-application of 3 μM SB-334867 and 3 μM TCS OX2 29 (open circles) compared to control (filled circles). B) Application of 3 μM SB-408124 (open circles) compared to control (filled circles). Data presented are mean values ± SE. * indicates a statistically significant difference (paired *t*-test, $P < 0.05$) from values at pH 7.4.

Table 4.1 *Baseline hypoglossal burst parameters from WT and Lmx1b$^{f/f/p}$ rhythmic medullary slices*

Genotype	*n*	Frequency (bursts/min)	Duration (sec)	CV IBI
Wildtype	19	9.2 ± 0.9	0.66 ± 0.4	0.28 ± 0.024
Lmx1b$^{f/f/p}$	21	6.8 ± 0.5	0.68 ± 0.2	0.44 ± 0.026*

Data are mean ± SE. A *t*-test (* $P < 0.001$) or Mann-Whitney rank sum test was used, as appropriate, to determine a statistically significant difference from WT.

Table 4.2 *Effects of hypocretin receptor antagonism on the pH response in WT and Lmx1b$^{f/f/p}$ rhythmic medullary slices*

Genotype	*n*	Treatment	Burst duration at pH 6.9 as a percentage of control	Burst amplitude at pH 6.9 as a percentage of control
Wildtype	6	SB-334867 + TCS OX2 29	100 ± 10	93 ± 3
	6	SB-408124	107 ± 7	93 ± 6
Lmx1b$^{f/f/p}$	7	SB-334867 + TCS OX2 29	120 ± 7*	100 ± 4
	6	SB-408124	119 ± 3	83 ± 12

Data are mean ± SE. A paired t-test was used to determine statistical significance of values at pH 6.9 compared to baseline (pH 7.4). * $P < 0.05$

4.5. References

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Chapter 5 General discussion

5.1. Using reduced preparations to examine neuroventilatory modulation

Central CO_2 chemosensitivity has been extensively investigated in a wide variety of animal preparations. Since Mitchell et al. (1963) first discovered central chemosensitive neurons on the ventral medulla of the cat, the identification of populations of specific chemoreceptors and their interactions have been made using preparations ranging from isolated neurons to intact animals (Nattie and Li 2001; Wang et al. 1998). Throughout my dissertation, I investigated neuroventilatory CO_2 sensitivity using the arterially perfused *in situ* preparation and the *in vitro* rhythmic medullary slice preparation.

5.1.1. The in situ preparation as a tool to examine effects of CO_2 on ventilation

Much of the work in this dissertation utilized a rat model, the arterially perfused *in situ* juvenile rat brainstem preparation, as a means to study the hypercapnic ventilatory response. This preparation is advantageous as it maintains the complexity of respiratory networks in an unanesthetized animal. This is important in that previous research has been conducted primarily on reduced preparations (cell culture and brainstem slices) or in the presence of anesthetic agents, which have potential confounding influences. The perfused brain reduces trauma to the central nervous system and peripheral nerves during surgery, as compared to more reduced preparations such as acute slices. The brain also is less likely to suffer from ischemia than more reduced preparations as the dissection time is fast and the aorta is cannulated quickly to perfuse the brain with artificial cerebrospinal fluid (aCSF) through the normal vasculature. Also a key benefit to using the *in situ* preparation is that it maintains integrative function of central networks. Less reduced preparations, such as those studied *in vivo*, are potentially compromised by anesthesia, which is known to blunt the hypercapnic chemoresponse (Martin-Body and Sinclair 1985). Protocols using the *in situ* preparation are carried out in the absence of anesthesia as pain perception is abolished by decerebration. This preparation also permits easy

pharmacological manipulation and control of external variables (such as temperature, CO_2 and O_2 levels).

My data showed that this preparation exhibited CO_2 sensitivity that is intermediate to more intact animals and more reduced preparations like brainstem slices. Several datasets showed the *in situ* juvenile rat preparation was responsive to both mild (7%) and severe (9-10%) hypercapnia, as determined by significant changes in phrenic nerve output (Chapters 2 and 3). This is in agreement with responses published by other investigators (Day and Wilson 2005, 2007; St-John and Paton 2000). The responses I observed in phrenic burst frequency and amplitude in response to hypercapnia in the *in situ* preparation were dampened compared to ventilatory responses observed *in vivo*. This was to be expected, however, as many influences known to facilitate ventilation and chemosensitivity, but originating outside the brainstem, were absent from my preparations. The *in situ* preparations have deactivated peripheral chemoreceptors, are vagotomized, decerebrate, and slightly hypothermic - all factors known to reduce the hypercapnic ventilatory response (Gautier et al. 1993; Hayashi and Sinclair 1991; Martin-Body and Sinclair 1987; Maskrey 1990; Nattie et al. 1991).

5.1.2. The in vitro slice preparation as a tool to examine mechanisms and modulation of neuroventilation

The *in vitro* medullary slice preparation provided investigative benefits by targeting a limited section of nuclei within the brainstem. While hypoglossal rhythmic activity recorded in these preparations may not represent eupnea directly (St-John 1996), medullary preparations allowed precise manipulations of elements within the slice. This gave me the ability to investigate putative rhythmogenic elements located in the slice, in the absence of external influences. While I must be cautious when extrapolating results to the whole animal, data from *in vitro* slices provide information indicating actions central in origin.

5.1.3. The utility of slices derived from genetically modified mice

I also used *in vitro* slice preparations derived from knockout mice lacking all central serotonin neurons (*Lmx1b*$^{f/f/p}$ mice; Ding et al. 2003; Zhao et al. 2006). The *Lmx1b*$^{f/f/p}$ knockout mouse facilitated investigation of 5-HT neurons in neuroventilation and responses to acidosis. This genetic alteration of central 5-HT neuron expression provides targeted and specific deletion of 5-HT neurons (>99%). Other studies using pharmacological deletion or inhibition of 5-HT neurons do not impact the system to such an extent (Messier et al. 2002, 2004; Nattie et al. 2004; Taylor et al. 2005). Although the *in vitro* slice preparation is limited to use in neonates, this is beneficial as many of the respiratory abnormalities in the *Lmx1b*$^{f/f/p}$ mice disappear with age due to compensatory mechanisms occurring during development (Hodges et al. 2008, 2009). Using *in vitro* slices derived from neonatal *Lmx1b*$^{f/f/p}$ mice illustrates the roles of 5-HT neurons in normal respiratory function prior to this potential compensation.

5.2. Neurotransmitter systems that contribute to central chemosensitivity

5.2.1. Role of 5-HT system in central respiratory chemosensitivity

Results from my experiments have documented the critical nature of 5-HT neurons and identified the post-synaptic 5-HT mechanism that promotes ventilation during hypercapnia. Previous research proposes the importance of 5-HT neurons themselves (and thus 5-HT as an important modulator) in the hypercapnic ventilatory response (Hodges et al. 2008; Messier et al. 2004; Nattie et al. 2004; Taylor et al. 2005). However, none have shown which post-synaptic receptor subtype is primarily responsible for the augmentation in phrenic nerve output in an intact system. I clearly demonstrated that 5-HT neuron activation and activation of 5-HT$_2$ receptors were critical in producing both the frequency and amplitude increases in phrenic bursts associated with hypercapnia (Fig. 2.5).

I also re-examined the role of 5-HT neurons themselves in central chemosensitivity. This was achieved in two ways: 1) by inhibiting 5-HT neurons with 8-OH-DPAT in the *in situ* rat preparation and evaluating the response to systemic

hypercapnia, and 2) by comparing the response to changes in pH in medullary slice preparations from wildtype (WT; containing 5-HT neurons) and *Lmx1b*$^{f/f/p}$ mice (which lack central 5-HT neurons) (Ding et al. 2003; Zhao et al. 2006). The phrenic burst frequency response normally associated with elevated CO_2 in the *in situ* preparation was not observed when 8-OH-DPAT was added to the perfusate (Fig. 2.4). These results illustrated that an 8-OH-DPAT-sensitive process was critical for the hypercapnic response. 8-OH-DPAT activates 5-HT_{1A} autoreceptors on 5-HT neurons, hyperpolarizing and effectively silencing these cells, and reducing their release of neurotransmitters (McCall and Clement 1989; Veasey et al. 1995). My results support a conclusion that 5-HT neuron activity is critical for chemosensitivity.

In addition to the *in situ* rat preparation, I made use of the transgenic *Lmx1b*$^{f/f/p}$ mouse in my investigation of the role of 5-HT neurons in ventilation and chemosensitivity. In a neonatal *in vitro* rhythmic medullary slice preparation, I demonstrated that WT responded to acidosis (pH 6.9) by significantly increasing hypoglossal burst frequency, while this response was absent in *Lmx1b*$^{f/f/p}$ (Fig. 4.1). As the normal chemoreflex is absent in the system lacking 5-HT neurons, I conclude that such neurons are again critical for chemosensitivity. Both results from the *in situ* and *in vitro* experiments are complementary and support a conclusion that 5-HT neurons are critical in central chemosensitivity.

5.2.2. Role of hypocretinergic system in central respiratory chemosensitivity

Data suggest that hypocretin-synthesizing neurons may themselves be chemosensitive, increasing their firing rate in response to CO_2 (Sunananga et al. 2009). Also, hypocretins appear to modulate both ventilation and chemosensitivity, particularly during wakefulness (Dias et al. 2009; Nakamura et al. 2007). Some of the mechanisms involved in such modulation may be associated with non-5-HT neurons located outside the raphé (Dias et al. 2009). Results from the *in situ* preparation indicated that exogenous hypocretin application had little or no effect on ventilation or chemosensitivity (Fig. 3.1). I concluded from this that either hypocretins had no influence or that endogenous hypocretin was sufficient. As both baseline ventilation and chemosensitivity were

preserved, my data do not support a role of hypothalamic hypocretin-synthesizing neurons as important chemoreceptors. Both the *in vitro* rat and mouse slices were relatively insensitive to exogenous hypocretin (Fig. 3.1, Fig. 4.3). In particular, this treatment did not emulate the change in burst frequency in the mouse slice associated with a response to acidosis (Fig. 4.1). These data further refute the role of hypocretin in chemosensitive neurotransmission.

In the *in situ* preparation, chemosensitivity was not observed when endogenous hypocretin receptor activation was blocked. Thus, endogenous hypocretin facilitated central chemosensitivity through activation of hypocretin receptors type 1 (hcrt-r1; Fig. 3.2). I conclude that, although hypocretin neurons may not play a direct role in chemosensation, hypocretin neuropeptides provide a critical facilitation necessary for chemosensitive mechanisms to function. Curiously, antagonism of hypocretin receptors in *in vitro* mouse slices did not impact the increase in hypoglossal burst activity associated with acidosis (Fig. 4.4). Clearly, this *in vitro* slice was capable of generating a chemosensitive response without hypocretin receptor activation. In contrast, hypocretin receptor activation was critical for central chemosensitivity of the *in situ* rat preparation. The cause of disparity regarding the role of hypocretin in central chemosensitivity in these two preparations has not been identified. This disparity could result from a difference between the species (mouse vs rat), the level of maturity of the preparations (neonatal vs juvenile rat), or the degree of reduction (*in vitro* brainstem slice vs *in situ* arterially perfused brainstem). Understanding these differences is an obvious future direction for this research.

5.3. Respiratory control-related interaction between the serotonergic and hypocretinergic systems

Growing evidence suggests that central chemoreception is achieved by multiple potential interaction sites within the brain (Nattie and Li 2009). A recent publication shows a significant link between hypocretins and chemoreception in the retrotrapezoid nucleus and between hypocretins and 5-HT neuron function in the midbrain raphé

(Brown et al. 2001; Dias et al. 2009). The anatomy for a similar interaction between hypocretins and 5-HT neurons exists since hypocretin receptors 1 and 2 are present in the medullary raphé, and projections of hypocretin neurons terminate in that area (Marcus et al. 2001; Nambu et al. 1999; Zheng et al. 2005). My results showed a functional interaction between hypocretins and 5-HT neurons. I compared the effects of hypocretin receptor antagonists on baseline hypoglossal burst parameters in rhythmic slices derived from both WT and *Lmx1b*$^{f/f/p}$ mice. Blocking endogenous hypocretin significantly decreased burst frequency in preparations from both genotypes; however, this depression was more severe in WT preparations (Fig. 4.3). This indicates not only that hypocretin was facilitating respiratory-related nerve discharge, but also that part of this modulation was through activation of 5-HT neurons.

5.4. Ventilatory chemosensitivity and Sudden Infant Death Syndrome (SIDS)

I have shown that an experimentally induced dysfunction of the 5-HT system compromised critical homeostatic reflexes. If such dysfunction occurred in an infant, vulnerability to SIDS would be enhanced. Furthermore, the medullary 5-HT systems of humans and rats are sufficiently similar, and ample evidence of 5-HT anomalies among SIDS victims (Kinney et al. 2001, 2003; Kinney 2005) shows that 5-HT dysfunction can be considered a likely cause of at least a subset of SIDS cases. The implications of this causational understanding of SIDS is that diagnostic and therapeutic tools aimed at SIDS might be well advised to target the 5-HT system.

5.4.1. The medullary 5-HT system in humans and rats

The medullary 5-HT system consists of 5-HT-containing neurons located in the midline raphé (raphé pallidus, raphé magnus, and raphé obscurus), the lateral extraraphé, and the ventral surface of the medulla (Kinney et al. 2001; Paterson et al., 2006b; Richerson 2004). In humans, central 5-HT neurons are in homologous regions to those in rodent brainstems (Paterson et al. 2009). However, an additional component is defined in the human system, the arcuate nucleus (Kinney 2005). This nucleus is positionally analogous to 5-HT and glutamatergic neurons of the ventral medullary surface in rats

(Paterson et al. 2006a). In rodents, these neurons are chemosensitive and this appears to be true for neurons in the arcuate nucleus as well. Functional neuroimaging of adults breathing elevated CO_2 shows an increase in firing in this area (Gozal et al. 1994). Also, the arcuate nucleus was absent in the autopsied brain of an infant who was insensitive to CO_2 (Folgering et al. 1979). Additionally, tracing studies show that 5-HT neurons in the human medullary raphé project to respiratory-related nuclei such as the nucleus tractus solitarius (NTS) and hypoglossal nuclei (Paterson et al. 2009). Similar projections are observed in rodents (Dahlstrom and Fuxe 1964; Jelev et al. 2001). The medullary 5-HT system of humans and rats, thus, seems functionally and anatomically disposed to playing a significant role in respiratory CO_2 chemosensitivity.

Furthermore, consistent distributions of 5-HT_{2A} receptors have been described in the medulla of humans and rats in nuclei critical for respiratory control (Paterson and Darnall 2009). 5-HT_{1A} receptors co-localize primarily with 5-HT neuronal cell bodies in the human, which is similar to the rodent distribution (Paterson et al. 2006a).

5.4.2. Medullary 5-HT dysfunction in SIDS brainstems and the relevance of current findings

The medullary 5-HT system is abnormal in ~50% of SIDS cases (Kinney et al. 2001, 2003; Kinney 2005). 5-HT receptor binding densities are lower in the arcuate nucleus and the raphé obscurus in SIDS cases. Specifically 5-HT_{1A} and 5-HT_{2A} receptor expression is significantly lower in SIDS cases compared to age-matched controls (Paterson et al. 2006b). Additionally, the number and density of 5-HT neurons is significantly higher in SIDS cases in regions of the raphé; however, many of these neurons are immature (Paterson et al. 2006b). Such abnormalities do not indicate if there is an excess or a deficit of available 5-HT. Recent evidence, however, suggests that the level of available 5-HT in SIDS versus normal brainstems is lower (Broadbelt et al. 2009). Collectively, these findings suggest that the 5-HT dysfunction, which putatively underlies SIDS, is based on receptor dysfunction and a deficiency in 5-HT production.

If I compare my experimentally induced abnormalities with those found in SIDS brainstems, I can postulate how a dysfunction in the 5-HT system, in addition to an

exogenous stressor such as hypercapnia, may contribute to SIDS. Complete inhibition of 5-HT_2 receptors with ketanserin eliminated the neuroventilatory response to CO_2 in the *in situ* rat preparation (Fig. 2.5). If I evaluate this result with the decrease in 5-HT_{2A} receptors found in SIDS cases, I can speculate that such a decrease may blunt the hypercapnic ventilatory response, a critical homeostatic reflex whose dysfunction could result in death. Additionally, I presented physiological evidence that indicated the importance of 5-HT neurons and 5-HT release in central chemosensitivity (i.e. 8-OH-DPAT eliminated the hypercapnic response *in situ* and the response to acidosis in medullary slices derived from $Lmx1b^{f/f/p}$ mice was absent) (Fig. 2.4, Fig. 4.1). The recently reported decrease in 5-HT levels in SIDS cases could reflect a diminished ability to respond to CO_2 as an exogenous stressor.

A role for 5-HT_{2A} receptors in respiratory CO_2 chemosensitivity and a developmental change in the expression of this receptor subtype may combine to provide the third component of the triple-risk model for SIDS. In addition to an underlying vulnerability and an exogenous stressor, SIDS occurs during a critical developmental period of homeostatic control (Filiano and Kinney 1994). I provided evidence for the importance of 5-HT_2 receptors in the response to hypercapnia (Fig. 2.5). A significant reduction in the expression of this receptor subtype is reported in several respiratory-related nuclei in rats at post-natal day 12, after which expression increases again (Liu and Wong-Riley 2009). Thus it is possible that this reduction in the function of the 5-HT system during such a critical period may render the animal naturally and transiently less able to respond to an exogenous stressor, such as hypercapnia. If a dysfunction in the 5-HT system is superimposed onto this transient period of reduced function, the ability to respond to hypercapnia may be severely reduced.

5.5. Summary

The 5-HT system of the medullary raphé is critical in central chemosensitivity. Using both the *in situ* brainstem preparation derived from rats and the *in vitro* medullary slice preparation from mice, I verified that 5-HT neurons were critical in generating a

response to CO_2, and that release of 5-HT from neurons activated by hypercapnia facilitated the respiratory rhythm through 5-HT_2 receptors. Hypocretins played a significant role in the neuroventilatory response to CO_2, primarily through activation of hcrt-r1, which may mediate the activity of 5-HT neurons. Medullary slice preparations from mice indicated that hypocretins mediate the hypoglossal burst duration response to CO_2 via 5-HT mechanisms. I also found that hypocretins facilitated baseline neural ventilatory output in part through 5-HT neurons. Thus, both the 5-HT and hypocretinergic systems are involved in modulating ventilation and hypercapnic ventilatory responses.

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Appendix

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Hi Andrea,

I am writing to acknowledge that I am aware that you will include material in your thesis that will also be published with me as an author in later papers, and that you will be the first author on those papers. Good luck with finishing your thesis.

Best wishes,
George